STUDIES ON PHENOLIC CONSTITUENTS OF SOME LEGUME FODDER TREES.

THESIS

SUBMITTED TO THE

BUNDELKHAND UNIVERSITY JHANSI

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BY

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UNDER THE SUPERVISION OF
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जिस माँ के आशीषों से

जीवन के सुख अर्जित है।

उसकी पावन स्मृति में

यह मेरा शोध समर्पित है।

CONTENTS

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CERTIFICATE

It is certified that the thesis entitled " **Studies on phenolic constituents of some legume fodder trees**" is an original research work done by Smt Surabhi Yadav, M.Sc., M.Phill. under my supervision and guidance for the degree of Doctor of Philosophy in Chemistry, Bundelkhand University, Jhansi(UP).

I, further certify that :

- It embodies the original work of candidate herself.
- It is up to required standard both in respect of content and literary presentation for being referred to the examiners.
- The candidate has worked under my supervision for the required period at Indian Grassland Fodder and Agroforestry Research Institute, Jhansi.

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DECLARATION

I, Surabhi Yadav`, M.Sc., M.Phill, solemnly declare that with the exception of guidance and suggestion received from my supervisor Dr B. K. Bhadoria, Principal Scientist (Organic Chemistry) Indian Grassland, Fodder and Agroforestry Research Institute, Jhansi the work incorporated in the thesis entitled " **Studies on phenolic constituents of some legume fodder trees**" is an original research work and out come of my own efforts. To the best of my knowledge, any or whole of thesis has not been submitted for a degree or any other qualification of any University or examining body in India/elsewhere

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SUMMARY

The limited land resources for forage and food production due to geometrical increase in Indian livestock and human population seriously indicates that agricultural production need to be intensified, if current standard human and animal nutrition is to be maintained. Farming system in country normally include small scale crop and the livestock. The performance and productivity of animals are generally poor because of limited cultivation and meager feed resources, especially during dry season. Shrubs and trees are one solution for this vulnerable problem, as they cater the need of animal survival in term of protein, energy mineral and vitamins. Besides these they also produce variety of chemicals as a by product of primary metabolism, many times which act as weapons for their defense, against herbivores/pathogens. Such secondary metabolites are also often referred as anti nutritional factors for animals. The secondary metabolites play key role in the utilization pattern of herbages.

Plant phenolics constitutes by far the largest and most wide spread group of secondary metabolites. The utility of leguminous trees as high quality fodder cannot be ruled out but keeping in view the anti nutritional nature of phenolics the present study has been under taken on following leguminous trees.

1. *Albizia procera (Roxb.) Benth*
2. *Bauhinia purpurea (Linn.)*
3. *Leucaena diversifolia (Schltdl.)*

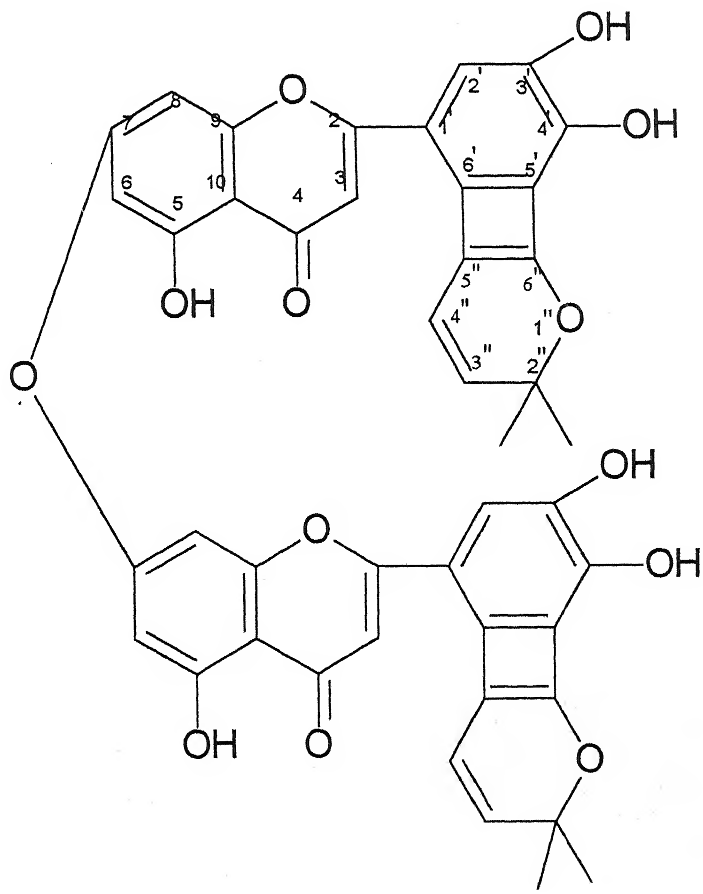
Chapter I :-

The first introductory chapter is an comprehensive account on plant phenolics, since very beginning , their relevance in the plant animal interaction,

diversity in chemical nature, ecological and nutritional importance with special reference to anti nutritional nature owing to their affinity towards protein.

Chapter II :– Isolation and characterization of flavonoids from the leaves of *Albizia procera* .

This chapter incorporates the studies of three new biflavanoids characterized as **Bis dimethyl chromene [5',6' : 5'',6''] 7-O-7 bil-uteolin (I). (7-3'' : 7-7'') Biluteolin 1'',5'' dihydroxy anthraquinone** (II). **I-3', II-3', I-4', II-4', I-5, II-5, I-13, II-13 octahydroxy I,II [2'',3'' : 5',6'] difurano I-2', II-2' bis hydroxy methyl I,II bis hydroxy phenyl [7,8 : 15,16] I-6,II-6 biflavonoid** (III).



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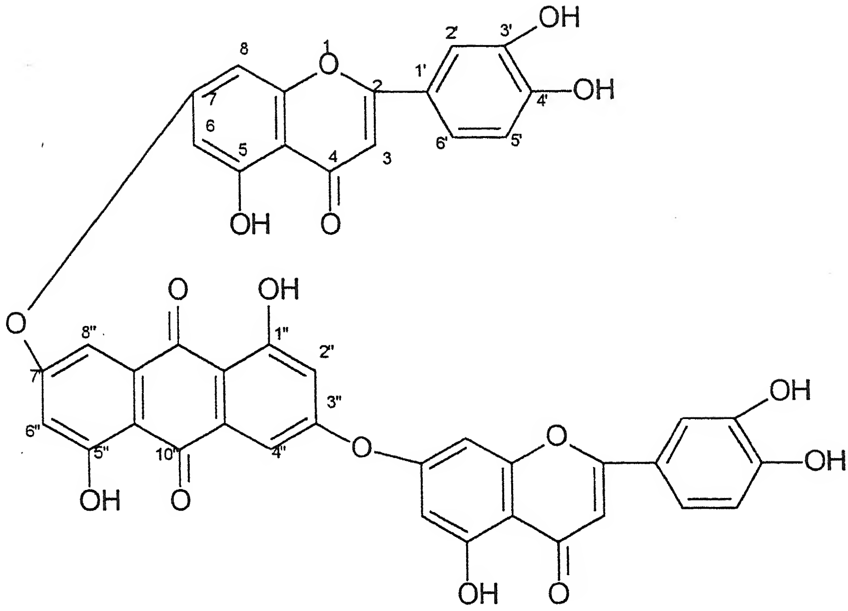

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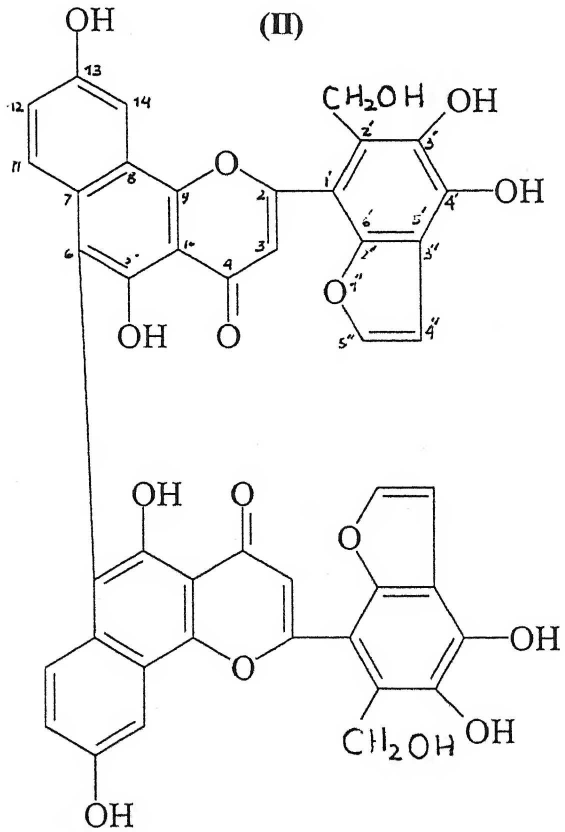

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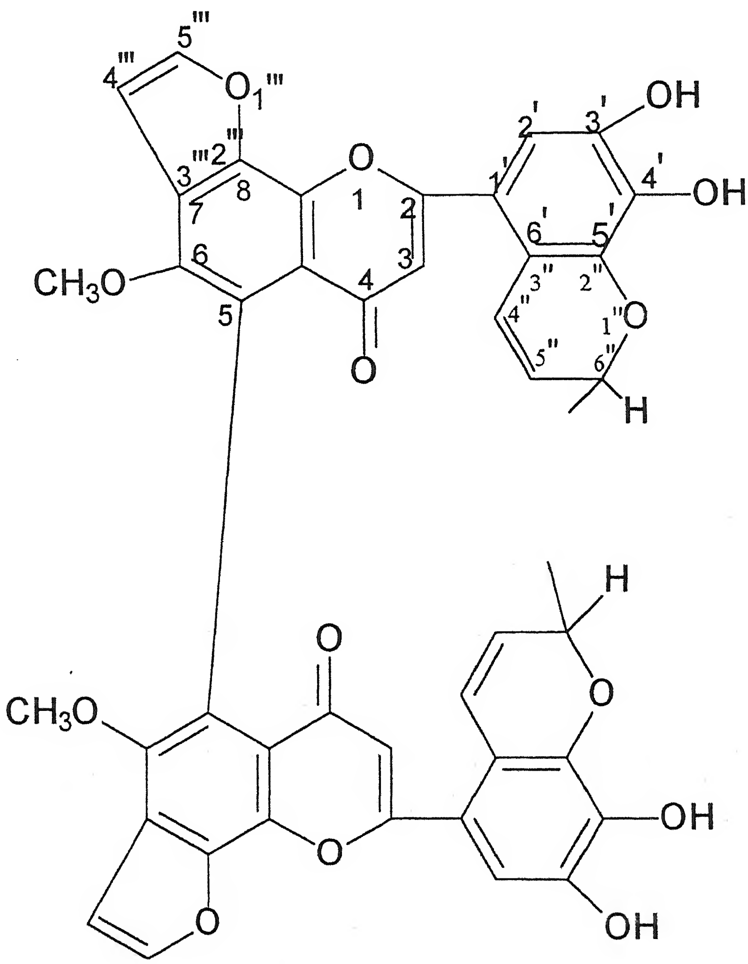
Chapter III :– Isolation and characterization of biflavonoids , from the leaves of *Bauhinia purpurea.* The leaves of *Bauhinia purpurea* yielded **I-3',4', II-3',II-4', tetrahydroxy I-6 , II-6, dimethoxy I,II difurano [2''',3''' : 7,8] I,II bis monomethyl chromene [2'',3'' : 5'6'] I-5, II-5 biflavonoid** (IV). **I-4',II-4' dihydroxy I-7, II-7 dimethyl I-3-α–L-rhamnoside II-3-α–D glucoside I-5, II-5 biflavonide** by using recent techniques of spectroscopy (V).



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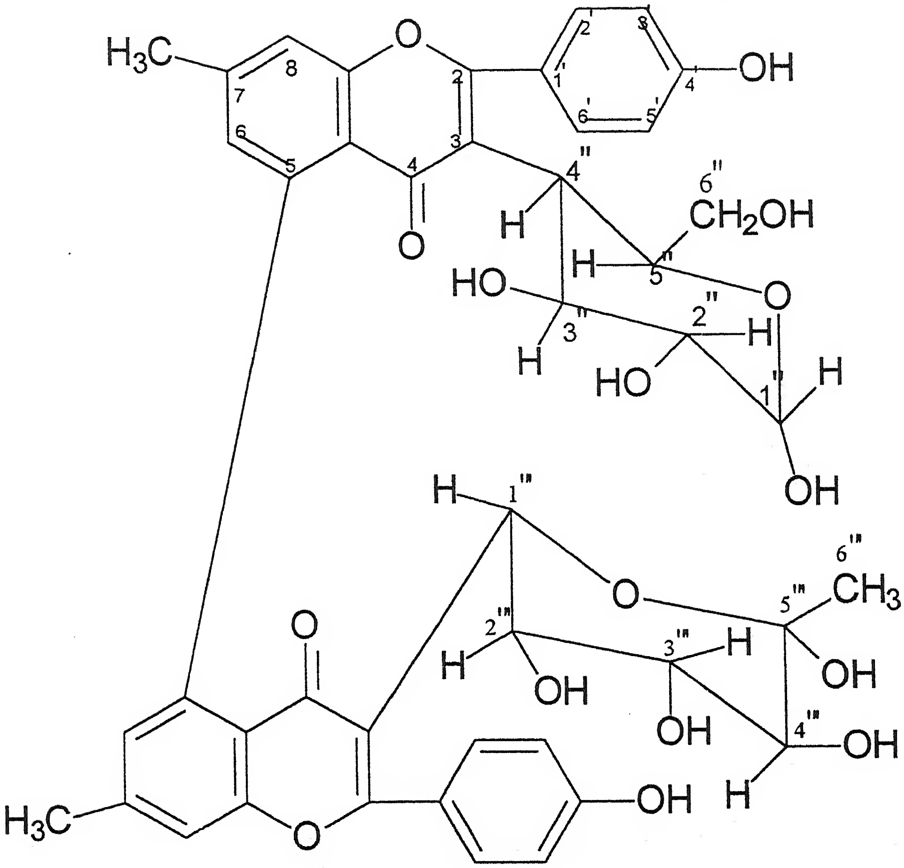
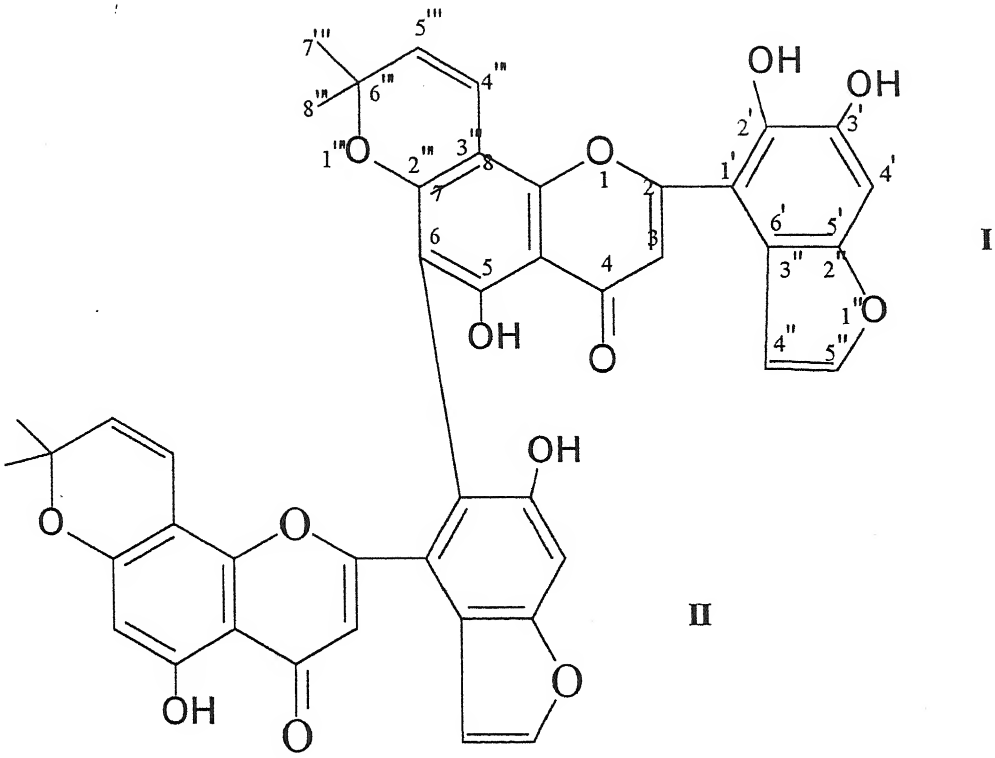


(V)

CHAPTER IV – Isolation and characterization of the biflavonoid from the leaves of *Leucaena diversifolia*..

The *Leucaena diversifolia* leaves afforded **I-2',I-3', II-3',I-5, II-5 pentahydroxy I,II [2''',3''' : 7,8] bis dimethyl chromene I,II [2'',3'' : 5',6'] difurano I-6,II-2' biflavanoid** (VI).



(VI)

CHAPTER V :– Chemical and Biochemical assessment of leaves of *Albizia procera*, *Bauhinia purpurea*, and *Leucaena diversifolia.*

On nutritional evaluation of the fodder tree leaves under investigation viz *Albizia procera, Bauhinia purpurea* and *Leucaena diversifolia* revealed that the nutritional attributes were in adequate quantities for the ruminants. The IVDMD (*in vitro* dry matter digestibility) data revealed that except *Bauhinia purpurea* the *Leucaena diversifolia* and *Albizia procera* were moderately digestible.

Apart from this *Leucaena diversifolia* was found to contain mimosine an anti nutritional phenolic, non proteineous amino acid (1.711% DM basis).

CHAPTER VI :– Protein binding efficacy of biflavonoids. It deals the study of protein binding efficiency with BSA (fraction V) of relatively smaller polycyclic phenolic molecule isolated from plants leaves under investigation. The study led to infer variation in protein binding capacity was dependent of molecular size and water solubility.

It is much warranted that the tree leaves should be utilized wise fully with prior information of phenolics. The protein biflavonoid interaction is of ecological importance because the biflavonoids deposited on the plant leaves come in direct contact with proteineous enzyme secreted by invading insects resulting into deterrency. The information generated by the investigation is of much importance to ecologist to explain plant-insect interaction and animal nutritionist for inclusion the leaves in dietry system for compound feed and ration for dry season.

Chapter - I

Introduction

INTRODUCTION

The earliest of man's uses of plants material rich in polyphenolics were conversion of animal hides to leather and archeological records related to this in mediterranean region was around 1500 B.C. Studies of chemistry of natural products have been a dominated topic of large interest in organic chemistry for almost 200 years. In twentieth century with blooming of biochemistry, there came the realization that some natural products (eg. amino acids, fatty acids, nucleotides etc.) possessed distinctive role in life of all living beings. Their occurrence and biosynthetic pathway are similar, if not identical in most of the organism. They are generally referred as Primary metabolites. On the contrary, infinitely greater part of natural products of microbial and plant origin, such as terpenes, alkaloids, polyenes, polyacetylene, phenolics and mycotoxins occur in nature. Their presence indeed often constituents something of taxonomic explicit function in the economy of the producing organism and because this reason they are often collectively called Secondary metabolites [Beart *et.al.*, 1985].

None the less the question of function of secondary metabolite in plant and microorganism has provoked a continued debate and speculation. Two theories in this context were put forward one based on plant animal co-evolution [Frankel,1980, Ehrlich and Raven, 1965, Rhoades, 1979]. Another theory focuses, that secondary metabolites are produced in plants response to an environmental and ecological challenges as strategem of a chemical weaponry appropriate to the environmental pressure .

In this context the purported role of phenolics is widely quoted. Plant phenolics probably constitutes the largest group of plant secondary metabolites. The relevent physiological effect of phenolics is considered to be that of

astringency based on their ability to complex with proteineous substances. Feeny [1970] has speculated further on the function of polyphenols as characteristic of chemical defense of plants. They act as quantitative dosage dependent barriers to monogastric and polygastric herbivores.

Plant phenolics display tremendous variation in structure ranging from simple molecules containing a single aromatic to highly complex polymeric substances such as tannin and lignin. Besides the simple monocyclic phenolic acids, the larger number of plant phenolic compounds belong to the flavonoid group. They have a common basic structural unit made up of two benzene ring linked together by three carbon as C_6-C_3-C_6 skeleton. Variation in the state of oxidation of C_3 fragment produces diverse class of chemicals like catechin, diydrochalcones, flavone 3,4-diols, aurones, anthocyanins, dihydroflavonols, flavonols, isoflavonoids, coumstenes and pterocarpenoids etc. Flavonoids are characteristic of flowering and non flowering plants and presumably evolved during the Devonian period (350 mybp) [Swain and Cooper, 1980]. Simultaneous evolution of chemical weaponry system in plants with variable environmental pressure, which they face, led biosynthesis of biflavonoid in land plants abiding malonyl-co-enzyme pathway.

Presently the biflavonoids have been reported to occur in almost every class of plant kingdom except algae and fungi. The number of new biflavonoids in last 5-6 years is not very large. Among the Bryophytes *Hylocomium splendens* (Hedw.) B.S.G. was found to contain dihydroxyrobustaflavone and a biluteolin [Becker *et.al.*, 1986] and *Bryum capillare* (Hedw.) yielded bryoflavone and hetero bryoflavone [Geiger *et. al.*,1987]. In Pteridophyte the order Psilotales and Selaginellates were characterized by the presence of amentoflavones as the major biflavonoids. Wallace and Markham [1978], have reported a mixture of di and tri-*O*- glucosides from aeriel parts of *Psilotum nudion* and *Tmesipteris* spp. The

occurrence of amentoflavone derivative in many genera of Filicales [Wada *et.al.*, 1985] suggested that the ability to synthesize biflavone is plesiomorphic in the pteridophytes having been inherited from an ancient common ancestor of other biflavonoid containing common vascular plants.

The Gymnosperms are rich in biflavonoids, the family Cycadaceae is characterized by presence of amentoflavone and hinokiflavone and their derivatives [Jayaprakasam *et.al.*, 2000, Dossaji *et.al.*, 1975 a] while the family Zamiaceae contained only amentoflavone series . Their pattern was characterized by Mohammed *et.al.*, [1983], in 3 species of *Encephalartos* but Quasim *et.al.*, [1985b] reported the presence of hinokiflavone derivative from *Zamia angustifolia.* The leaves of living fossile *Ginkgo biloba* have been known for biflavones and were reported by Briancon-Schied *et.al.*, [1983]. Recently Ghosal *et.al.*, [2002] has added to amentoflavone 3-8 linked dimer. However the amentoflavone is of universal occurrence in Cupressaceae of Gymnosperm as a major component of leaves while robustaflavone has been detected sporadically as minor component in some families of Gymnosperm.

Biflavonoids have now been recorded in more than 32 genera belonging to 15 families of angiosperms as per records received by Dahlgren [1980]. The biflavonoids phenolics have commonly been reported in family Nandinaceae, Rhamnaceae, Euphorbiaceae, Thymeliaceae, Clusiaceae, Calophylloideae, Casurineace, Anacardiaceae, Caprifoliaceae and Leguminaceae. Flavonoids have been shown to possess several biological properties like hepatoprotective, anti-thrombotic, anti-inflammatory and antiviral activity due to their antioxidant and free radical scavenging activity [Saija *et.al.*, 1995]. The presence of associated sugar moiety affect the bioavailability of flavonoids sugar conjugated quercetin glycoside have been found to be absorbed in human system [Hollman *et.al.*,1999].

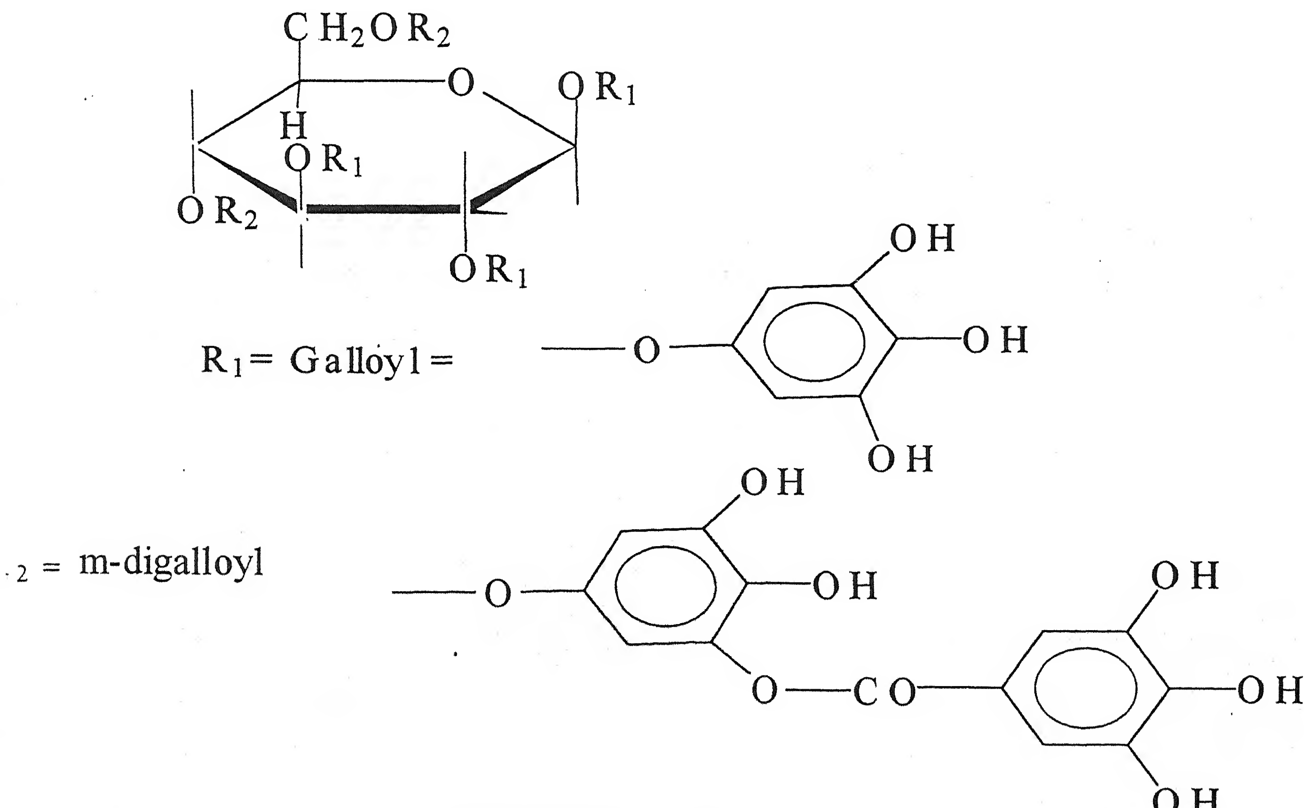
Dried roots of *Scutellaria baicalensis* containing flavonoids are used in China for the treatment of bronchitis, hepatitis, diarrhoea and tumour [Chiang, 1977,Tang *et.al.,* 1992] and have an inhibitory effect against human immunodeficiency virus HIV-I 3. Markham *et al.* [1988] reported rare biluteolin type biflavonol from *Dicranoloma robustam.* Kamil *et.al.,* [1987] reported biflavonoid from *Ochna pumila* and was reported to possess many medicinal properties. The roots of the plant is used as an antidotes to snake bites, menstrual disorders, asthma and have antitubercular activities .

Since several biflavone containing plants (*Viburnum runrifolium.* L., *Juniperus communis* L. and *Ginkgo bilobal* L.) are used medicinally. Pharmacological and biochemical tests have been carried out on a few biflavonoid.[Horhammer *et.al.,* 1967, Natarajan *et.al.,* 1970 , Ruckstuhl *et.al.,* 1979, Beretz *et al.* 1979 , Joly *et.al.,* 1980 a]. These test have included spasmolysis, peripheral vasodilatation, antibroadykinin activity, antispasmogenic action against prostaglandin, PGE , inhibition of cyclic GMP and cyclic AMP phosphodiesterase and inhibition of hepatoma cells . Further evidences of pharmacological effect of biflavonoids include their ability to inhibit the release of histamine [Amellal *et.al.,*1985] the adhesion of blood platelets [Cazenava *et.al.,* 1986] and the action of lens aldose reductase [Shimizu *et.al.,* 1984] to block the inflammatory effects of hepatotoxin [Iwu, 1985] and to act as a heart stimulant [Chakravarthy *et.al.,* 1981], Amentoflavone isolated from *Juniperus communis* L. has been shown to have strong antifungal properties causing strong inhibition of growth of *Aspergillus fumigatus*. In most of the cases the biflavonoid were proved to be more active than their monomer [Geiger and Quinn ,1982].

The biflavonoids deposited in plant leaves clearly come in direct contact with the enzymes secreted by invading pathogens. Natural function of

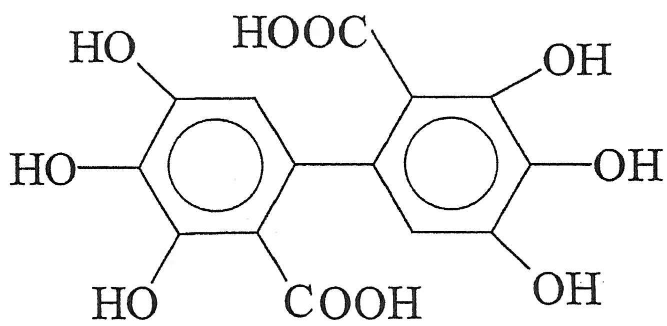
flavonoids were discussed briefly by Cody *et.al.*, [1986] in terms of factors influencing flavonoid evolution. An interesting feed deterrency activity of natural dihydrochalcone was described by Dreyer & Jones [1981]. Rozsa *et.al.*, [1982 b] found feeding deterrence along with insecticidal, anti parasitic, anti microbial and hypotensive activity by dihydrochalcone containing plant extract.

Tannins are also prominent class of compounds in phenolics, constituting the plant secondary metabolics. These are polyphenolics of varying moleuclar weights, occuring in all vascular plants. The presence of tannins have been correlated with the woodiness of the plant [Swain, 1963] or with the phylogenetic rank of the plant. [Hasalam, 1979, Haddock *et.al.*,1982]



Gallotannin **Fig. 1**

The polyphenolic interaction with protein underline a wide range of other apparently unrelated properties of plant materials, including ecological adaptation and astringency, chemical defense, fruits and floral pigmentation etc. Based on the chemical structures tannins are of two types hydrolysable and condensed tannin. Hydrolysable tannins are composed of ester of gallicacid (gallotannins Fig.-1) and ellagic acid (Ellagitannin Fig-2) with sugar core which is usually glucose.

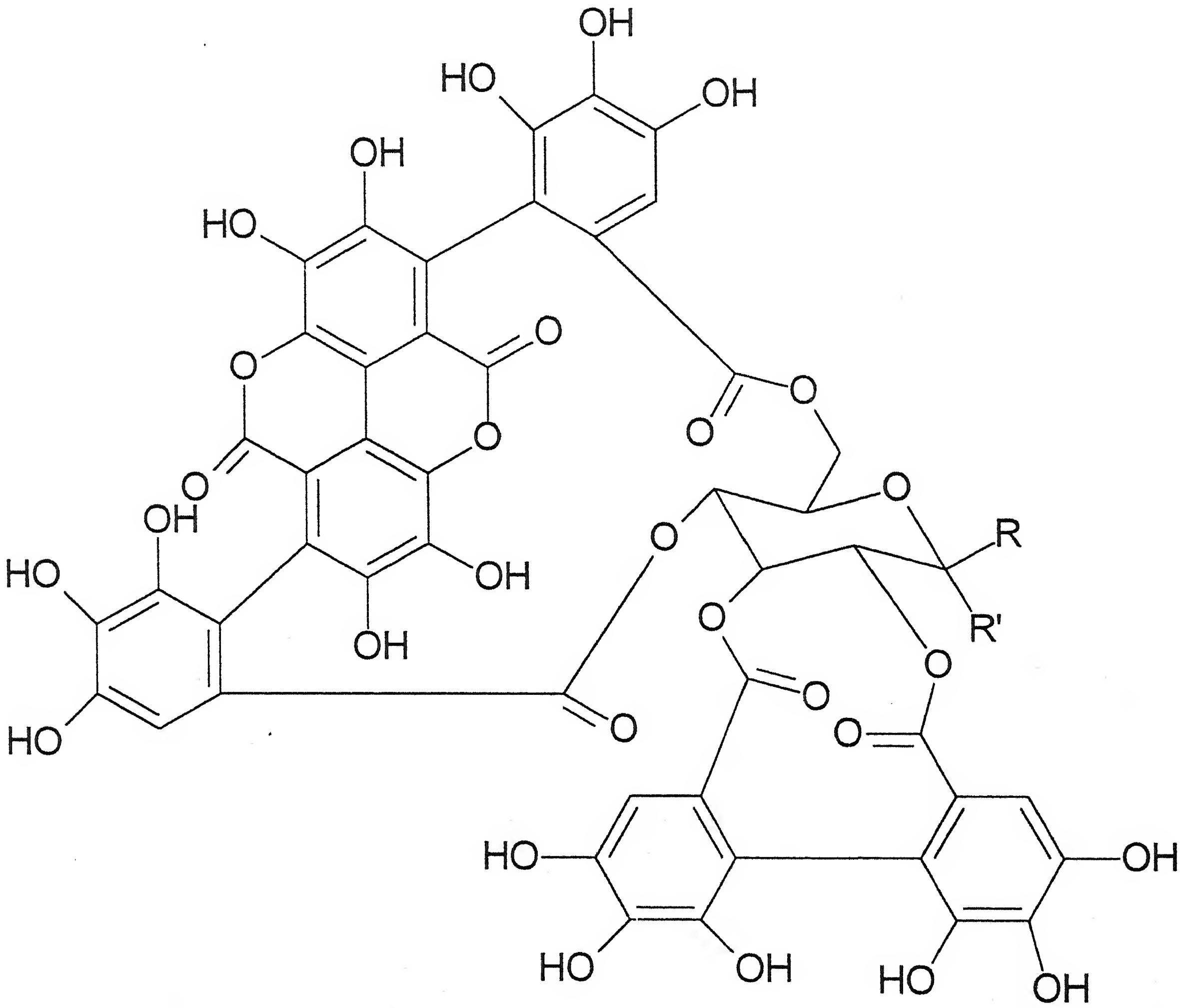


(Hexahydroxydiphenic acid)

Fig. 2

They are readily hydrolysable by acid or enzymes into monomeric products. Presence of hydrolysable tannin in plants effect adversely on herbivore nutrition by reducing intake, protein digestibility or by direct systematic toxicity [Kumar and Singh 1984 a]. The physiological effect of hydrolysable tannin depend on the nature of phenolic moiety present. Once the hydrolysable tannin

present in the diet is cleaved by the action of enzyme or suitable pH in gut, the released phenolic molecule is absorbed in blood stream and may create myriad of reactions depending on its nature. One of the interesting example is hepatoxic and nephrotic principles from *Terminalia oblongata* (Fam. Combretaceae), a deciduous shrub of Australia.



(I)	R=OH	R' = H	-	β Punicalagin
(II)	R = H	R' = OH	-	α Punicalagin

Fig - 3

The leaves of *Terminalia oblongata* were found contain 20-29% tannins. The shrub cause a disease "yellow fever" in the cattle and sheep and responsible for severe loss to the live stock wealth. [Brooks, 1964, Carroll, 1985]. The ingestion of leaves of *Terminalia oblongata* cause abdominal pain, photosensitization and dehydration [McCosker 1966 and Filippich *et.al.*, 1991]. The biochemical changes in blood and urine are indicative of liver and kidney damage [Everist, 1981]. The toxic principles isolated were α and β Punicalagin (Fig.- 3) and terminalin (Fig.-4) [Doig *et.al.*, 1990]

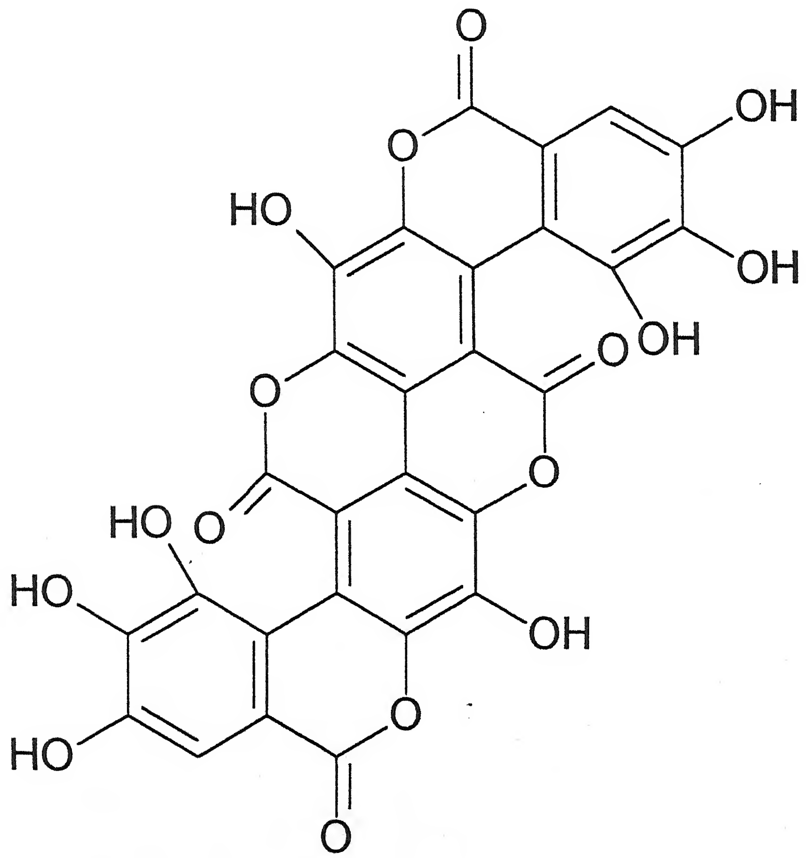
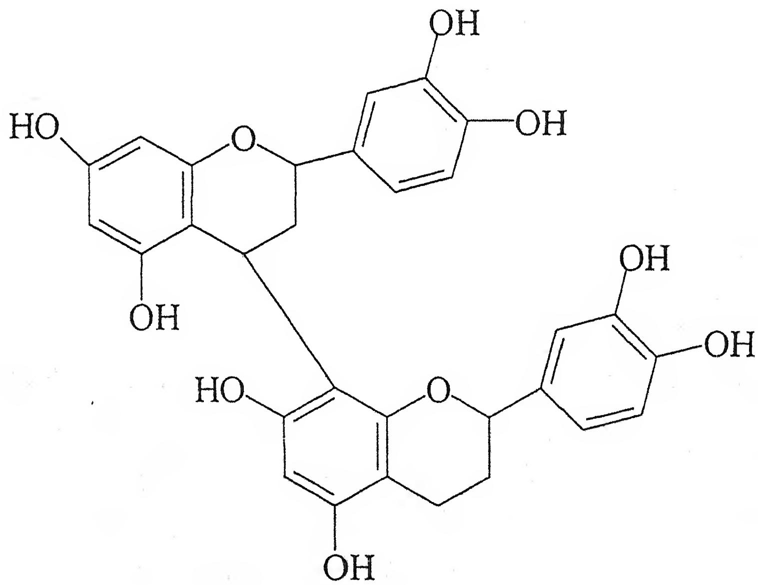


Fig. 4

The condensed tannins/proanthocyanidins are the most widespread and typical of the plant tannin. Bate Smith (1972,1973 b,1975,1977) surveyed a large number of plants for condensed and hydrolysable tannins. More recently tannins have been reported in some algae [Nishizawa *et.al.*, 1985]. Proanthocyanidins occur in numerous plant species encompasing many important plant derived for food materials and a number of economically significant forage legume.

The condensed tannins are usually sequestered in membrane bound vesicle in plant cell vacuole [Stafford, 1992, Klein *et.al.*, 2001]. The central vacuole is the large storage compartment for secondary metabolite. Condensed tannins are more correctly designated as proanthyocyanidin or polyflavonoids(Fig. 5) .



Condensed tannin

(Fig. 5)

Proanthocyanidins (PA) are derived from the polymerisation of flavon 3-ol units. Proanthocyanidin are phenylpropanoid polyphenol and are categorized by the type of monomer they contain either flavon 3-ol or flavan 3-4 diol or leucoanthocyanidin [Horvath 1981, Koupai – Abyazani *et.al.*, 1992]. Mostly the flavon – 3-ol monomer unit can be linked by C_4/C_9 or C_4/C_6 interflavonyl linkage which effect on the condensed tannin polymer molecule structure [Ferreira *et.al.*,1992, Porter, 1994]. The number of monomeric unit can vary, these can then produce an infinite variety of chemical structure which in turn effect the biological properties of the condensed tannin. The presence of proanthocyanidin in feed reduce the palatability, cause processing problem such as haze formation and decolorization. While the presence of proanthocyanidin has been traditionaly viewed as generally undesirable with antinutritional character [Jones *et.al.*,1976, Foo *et.al.*,1982, Sakar, 1976] associated with decreased palatability, digestibility by imobilizing bacterial enzyme or forming indigestible complexes with cell wall carbohydrates [Foo and Porter, 1980, Terril *et.al.*, 1992, Singleton and Kratzer 1973]. The antinutritional character of proanthocyanidin is certainly true when PA are present above 5% on dry matter basis, however presence in low level in feeds prevents bloat in cattle (Lee *et.al.*, 1996, Waghron *et.al.*, 1987)when given in diet with high soluble protein content. It is now recognized that low concentration of proanthocyanidin in ruminant can also be beneficial in many other ways such as reducing the effect of parasite in gastro intestinal tracts [Niezen *et.al.*, 1994] reducing the problem of fly strike [Niezen *et.al.*, 1998] and paradoxically by improving protein availability [Waghorn *et.al.*, 1994, Singh and Bhat, 2002]. Proanthocyanidin modify the microbial degradation of protein as well as reducing the activity of microbial enzymes[Barry,1989, Reed, 1995]. In the ecological literature, foliar tannins are traditionally viewed as precipitator increase the herbivore resistance of plants [Feeny, 1970. Barry and Dunkan,

1984]. The risk to unconfined animals having a choice of diets seems very small, but this is not so when high tannin food is the only choice several episodes of loss of live stock from eating tannins rich top feeds have occurred when little else is available [Wolter, 1974, Sandusky *et.al.*, 1977, Keeler *et.al.*,1978, Naser *et.al.*, 1982]. Hegarty *et.al.*, [1985] pointed out that phenolic compounds have been associated with animal death. Both the condensed tannin and hydrolysable tannins can exert toxic effect.

Proanthocyanidin/condensed tannin has been found to have greater affinity for dietery protein, salivary protein, digestive enzymes and carbohydrate which has been attributed to its strong hydrogen bond affinity to the carbonyl oxygen of the peptide group [Russell *et.al.*, 1968 a]. Besides this the precipitation can be result of covalent bonding [Swain, 1979] ionic bonding [Gustavson,1956] or hydrophilic bonding [Oh *et.al.*, 1980].

This strong hydrogen bond affinity of peptide has been observed by the other comparative studies on fixation of tannins by various polypeptide substrates. The large numbers of phenolic group in proanthocyanidins/condensed tannins molecule provide many points of attachment with favourable steric opportunities for linkage by hydrogen bonding with peptides of adjacent protein chains to form protein tannin complex. The fixation of tannin by hydrogen bonding has been shown to be independent of pH in the range of 2 to 8 but at higher pH, the fixation of tannin decline sharply because of the breaking of hydrogen bonding by formation of phenolic ion. Although the reaction of both tannin and protein depends on the spatial configuration of molecules and availability of phenolic group [Mangan,1988]. Yet the protein tannin interaction is most effective to pH close to the isoelectric point of protein. The molecular size of tannins is usually in the range 500-3000, requirement being that the tannin is soluble and small enough to orientate itself suitably between the protein chain,

but has sufficient phenolic groups to crosslink efficiently under suitable conditions of concentration and pH [Goldstein and Swain, 1963]. At the upper end of the molecular weight scale the phenolic polymers become less able to align themselves between protein components as they increases in size.

The enormous development in the field of phenolics has been mainly due to various advances in the analytical technique specially chromatographic separation technique and spectroscopic methods of analysis. The chromatographic techniques like TLC [Voirin and Jay, 1977, Kari *et.al.*, 1977, Edward, 1963] paper chromatography [Lederer and Lederer, 1957] column chromatography [Kari *et.al.*, 1977, Higuchi and Donellay, 1978] are still in frequent use.

Column chromatography still remains a very useful technique for preliminary seperation or purification, in large quantities from crude plant extracts. It has been discussed in depth by Markham and Mabry [1975]. Silica gel, polyamide, cellulose and Sephadex gel are excellent stationary phase adsorbents. On silica gel aglycone flavonoid have been separated with most commonly solvent chloroform : methanol or ethyl acetate : methanol mixture, whereas for glycoside it is advisable to add the water in solvent system. Polyamide is also used as packing material for column chromatography. Number of flavonoids have been separated on this adsorbent with the use of solvent system chloroform : methanol : methyl ethyl ketone (40 : 20 : 5) or with benzene : petroleum ether : methyl ethyl ketone : methanol (60 : 26 : 3.5 : 3.5). Numerous glycoside have also been isolated from crude extract with water : methanol or water : acetone mixture.

Thompson *et.al.*, (1972) showed usefulness of Sephadex LH-20 using alcoholic solvents, to separate mono, di and trimeric flavonols from variety of plant sources . More recently Nanaka and Nishioka's group has greatly extended

the range of separation achieved on Sephadex LH-20 by using aqueous methanol or ethanol [Nishioka, 1983]. Jones *et.al.*, [1976] used aqueous acetone 70% leaves extract of leguminious plant and chromatographed over Sephadex LH-20. These separations could further refined by juggling between Sephadex LH-20 and high porosity polystyrene MCI gel CHP - 20P.

Most chemical separation were carried out using a variety of techniques including column chromatography, paper chromatography and thin layer chromatography up to 1970. However these chromatography techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time pressure began to be used to decrease flow through time, thus reducing purification time of compound being isolated by column chromatography. High pressure liquid chromatography (HPLC) was developed in mid 1970's and quickly improved with the development of column packing materials, and additional convenience on line detectors. In the late 1970's new methods including reversed phase liquid chromatography allowed for improved separation between very similar compounds, use of computers and automation added to the convenience of HPLC. Additionally the substance can be separated quantified and identified in one system with UV diode array detector. There are several types of stationary phases available for the HPLC column. The most important of these are the so called chemically bonded stationary phases which are prepared by bonding organosilane molecules (eg. octadecyltrichlorosilane, octyltrichlorosilance or phenyltrichlorosilane) to the hydroxyl groups of silica type surface. Wehrli *et.al.*, [1978] reported that 50% of current separations are done, on octadecylsilyl bonded phase column commonly called as C_{18} column .

Acetonitrile-water mixture or methanol-water containing small amount of acetic acid are commonly used solvent system for phenolics. These mobile phases are suitable for UV detector and can easily be employed in gradient system in

complex separation. In many cases acetic acid has been replaced by phosphoric acid in mobile phase. Depending on the compounds to be separated it may be advantageous to replace C_{18} chemically bonded phase by C_8, RP-8 reverse phase material - a hydrocarbon polymer Zipax HCP-coupled with phosphate buffer-ethanol-ethyl acetate. Solvent system has also used for resolving hydrolysable tannins and related polyphenols. HPLC has been used recently to good effect. Beasley *et.al.*, [1977] have shown excellent separation of gallotannins extract with tetra hydrofuran and also of galloyl glucose core by methanolysis. Muller – Harvey *et.al.*, [1987] using HPLC and thin layer chromatography (TLC) were able to fractionate, characterize and measure complex mixture of condensed and hydrolysable tannins, flavanoids catechin, gallates and phenolic acids. Total phenolics were precipitated from extracts by ytterbium acetate after the method of Reed *et.al.*, [1985] and released from the precipitate for analysis by treatment with oxalates.

Thin layer chromatography (TLC) and paper chromatography remains most versatile method still for the detection and separation of phenolics from crude extract, the spray reagents for the detection of phenolics have been reviewed by Markham [1975]. Saleh [1976]observed that sodium ethoxide (0.05 M in ethanol) could be employed to differentiate isomeric forms of quercetin. For TLC on cellulose the classical solvent system 5-40% acetic acid, Butanol : Acetic acid : Water (4:1:5) or chloroform : Acetic Acid : Water (10:9:1) have been recommended [Markham and Wallace, 1979, Markham and Porter, 1979] . Recent advances shown that high performance thin layer chromatography (HPTLC) can provided better separation than TLC of complex mixture [Zlatkis and Kaiser, 1977] HPTLC is development of TLC using small particles, usually about 5 μm in diameter, all of a closely similar size.

UV spectroscopy is the most powerful techniques for identifying the phenolic type, for defining the oxygenation pattern and for determining the phenolic substituents [Mabry *et.al.*, 1970, Markham and Mabry, 1975, Markham, 1982, Wollenweber, 1982].

Electron impact Mass Spectrometry (EI-MS) serves as a valuable tool in determining the structure of phenolic, especially when small quantity of substance are available. Most of the phenolics are sufficiently isolated at probe temperature of 100-300^{0}C to allow mass spectroscopy without derivatization. The general rules of interpretation of molecular formula, type of substitution etc. have already given in depth by Markham and Mabry [1975]. Relative ion abundance and abundance ratio have been measured for 18 flavonoids by Madhusudanan *et.al.*, [1985] and for 10 polymethoxy flavone by Rizzi and Boeing [1984]. Field desorption Mass Spectrometery, [FDMS] the first ionization technique used for the study of thermolabile compounds without derivatization has been applied to the study of phenolics by Schulten and Games [1974], Zapesochnaya *et.al.*, [1984] and Domon and Hostettmann [1985]. Another soft ionization method has been developed is Desorption chemical ionization (DCIMS)which used a probe of electrically heated tungston source [Arpino and Devant, 1979. Hostettmann *et.al.*, 1981]. A third new technique is Fast Atomic bombardment Mass Spectra (FABMS) used for neutral atoms . Here a sample is dissolved in a polar matrix (e.g. glycol) with addition of trace amounts of inorganic salts (e.g. NaCl) to optimize production of cationic molecular ion. The deposited sample on copper target, is bombarded with energized atoms inducing desorption and ionisation. The ability of FABMS has been tested by various workers [Sakushima *et.al.*, 1984 De Koster *et. al.*, 1985, Domon and Hostett mann, 1985, Crow *et.al.*, 1986]. Self *et.al.*, [1986] have applied FABMS to plant phenols after fractionation by HPLC the FABMS of procyanidins of cider apples showed distinctive analytical

pattern. HPLC–FABMS applied to polyphenols of *Acacia nilotica* for separation and characterisation of number of flavanol mono and digallates.

^{1}H NMR or proton spectrometry is of tremendous utility for the judgement of structural environment, conformation, population trends of proton in molecule [Silverstein and Webster, 1998]. Carbon-13 with a natural abundance of only 1.1% and smaller magnet than ^{1}H, hence create weaker NMR signal, however with the advent in recent years of pulsed NMR and Fourier Transform analysis ^{13}C NMR spectroscopy has become more readily available . The dramatic increase for the ^{13}C NMR spectrometery for the assistance in structural elucidation, this has become a powerful tool in the study of phenolics. It should be stressed, however that ^{13}C NMR is in no sense superseding ^{1}H NMR but is complementary to it. Information gained relates to the carbon backbone of the molecule, where as ^{1}H NMR gives the proton environment of each protons. Although ^{1}H and ^{13}C NMR have become in modern age indispensable in organic chemistry by saving time and reducing the sample size, but these techniques have moved further for getting intrinsic picture of organic molecule. Many a times phenolic molecules contain diastereotropic hydrogen atoms and groups, and signal associated with them can be difficult to interpret using simple ^{1}H and ^{13}C NMR spectra. To achieve this, correlation spectroscopy (COSY) is used. Most of the two dimensional NMR (2D NMR) experiment fall in this category. The Cosy (correlation spectroscopy) involves, varied experiments depending upon requirement and conditions like ^{1}H-^{1}H COSY. ^{1}H-^{13}C COSY, HETCOR (Heteronuclear Chemical Shift Correlation) include HMQC (Heteronuclear Multiple Quantum Coherence) HMBC (Proton detected, Long-Range ^{1}H-^{13}C). The HMBC experiment capitalizes on these two and three bond coupling providing us with an extremely powerful spectrum. Here we get ^{1}H-^{13}C correlation and one is able to correlate quaternary carbon and nearby protons.

Beside these there are DQF COSY. TOSY, ROSY, INEPT and Gradient Field NMR to help the chemist for deciding precise and more precise molecular nature [Tamura *et.al.*, 1983, Bridle *et.al.*, 1984, Kondo *et.al.*, 1985].

Proanthocyanidins include a variety of chemical entries and this is reflected in a diversity of methods of qualitative analysis. Some of which have been used without adequate consideration of their limitation. Hide powder absorption, permanganate oxidation and Dennis – Folin "total phenol" reagent are example of non specific methods. The vanillin – HCl reagent of Bate-Smith [1954] is very sensitive and specific for condensed tannins of the flavan-3-ol and flavon –3,4 diol but non-tannin chalcone derivatives and monomeric flavonols can also reacts. On heating with dilute acid and n-butanol, proanthocyanidins are partially converted to the strongly coloured anthocyanidins. The reaction is only approximately quantitative, but is very specific and cyanidin, delphinidin, pelargonidin and other anthocyanidins are readily identified by paper chromatography and measured spectrophotometrically. The relative astringency method of Bate-Smith [1973 b] depends on precipitation of the whole protein by haemolysed blood (haemanalysis), and compared with standard amount of tannic acid or catechin. Results are expressed as tannic acid or catechin equivalents and give a useful measure of the protein precipitating activity of leaf powder or extracts. Marks *et.al.*, [1987] have used bovine serum albumin, γ-globulin, *β*-glucosidase (EC 3.1.1.2.1) and pancreatic protease under optimum condition to precipitate tannic acid and measured the protein in the precipitation by ninhydrin as an alternative to haemanalysis. Results indicate it is useful in binding studies with pure protein. In the method of Hagerman and Butler [1978] condensed and hydrolysable tannins in solution are precipitated by excess BSA and measured with ferric chloride. The $FeCl_3$ method was more sensitive to tannic acid. Bate-Smith [1954] used a combination of vanillin-HCL and positive identification of

the anthocyanidins after acid degradation. Jones *et.al.*, [1976] used aqueous acetone (700 ml/l) to extract >95% of the proanthocyanidin in frozen leaves of legume, followed by purification on Sephadex G_{50} column, vanillin-HCL was used to detect flavonol. The condensed tannin fraction was then rechromatographed on a column of Sephadex LH-20. Molecular weights were measured using an analytical ultracentrifuge and cyanidin : delphinidin ratio determined. The analytical methods described gives minimum information on molecular structure and many nutritional papers presently published are severely limited by the use of inadequate analytical methods.

Phenolics Protein interaction are potentially important phenomena in plant resistance to herbivores of all categories from arthopods to mammals [Harborne, 1992]. The role of phenolics in animal system is abnoxious as well as beneficial in relation to their molecular nature. Therefore, studies of these characteristic and not only of intrinsic scientific interest but they are of considerable practical significance for ecologist, nutritionist and agronomist.

The above preamble clearly transpire that there is enough scope for studies on phenolics from the leguminous fodder tree leaves, which constitutes major, dietery resources during the lean period to domestic ruminants.

However geometrical increase in the livestock population of India warrants that fodder and food production need to be intensified. Currently available resources can provide only 46.6% of total need to livestock population. However there is hardly scope of expansion in fodder cultivation due to pressure on agricultural land for food and cash crop. The forage resources in forests are also dwindling because of the explosive population situation. The deficit in fodder supply is further exacerbated with the availability of nutritionally low grade roughages as straw of high yielding dwarf varieties of cereals.

Rich in nutrient tree leaves constitutes a complementary fodder resources particularly during the lean period. Presence of anti nutritive constituents in tree leaves especially phenolics often limit their efficient utilization of nutrients in leaf fodder.Chemical investigation on tree leaves of native origin is an essential exercise in order to identify, species of highest nutritional value and low antinutritional factor. Therefore there is an imperative need to identify potentially important feed resources amongst trees and shrubs from native flora to explore the possibilities of including leaf fodder in the livestock dietry system. Following this time of rationale the *Albizia procera*, *Bauhinia purpurea*, *Leucaena diversifolia* have been selected for the studies on phenolics contituents which are well known for the antinutritional characters.

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Chapter - II

Isolation and characterization of flavonoids from the leaves of *Albizia procera*.

Albizia procera (Roxb) Benth. (Family Leguminosae) is commonly known as Safed sirish ,Modeloa, Bogai, Konda, Chikul, Tellachinta etc. [Jha, 1995] . *Albizia procera* is one of the important multipurpose tree of our country. It is found through out Assam, Bihar, Northern Madhya Pradesh, Andhra Pradesh, Central and Eastern Uttar Pradesh. It is one of the nitrogen fixing species. It is found growing on a variety of soils [Troup, 1986]. *Albizia procera* is large graceful sturdy tree and requires 37^0C to 46^0C for favourable growth, with a oval crown yellowish or greenish bark [Duthie,1962].

The leaf rachises are 25-46 cm long glabrous or pubescent with a large gland near the base. Flowers are yellowish white, sessile about 5-6 cm long with stemens a little more than twice the length of corrola. Pods are normally 10-20 cm by 1.8-2.5 cm glabrous brown, 8-12 seeds [Kanjilal, 1927]. The fruitining on the tree begins during April. The *Albizia procera* provides good quality charcoal and fuel wood and for high class furniture [Kanjilal, 1927]. Its fodder is valuable supplement to sheep and goat. The foliage are used as green manure and lopped fodder .

The genus *Albizia* is known for the presence of dihydroflavonol [Candy *et.al.*, 1978]. One of its species *Albizia amara* has been found to contain *trans p* coumaric acid and *cis p* coumaric acid, *trans* ferulic acid, myricetin, quercetin and apigenin. The deep survey of literature revealed the presence of dimethyl medicarpin in *Albizia procera* [Deshpandey and Shashtri, 1977].

The powdered leaves of *Albizia procera* were extracted with rectified spirit by cold percolation exhaustively and solvent was removed under vacuum. Dark coloured alcohol extractive was successively extracted with n-hexane, benzene, chloroform, ethyl acetate and acetone. The residual part was dissolved in 0.5% NaOH, and separated into alkali soluble and insoluble part. The alkali soluble

fraction was acidified and extracted with ethyl acetate, which was chromatographed over Si gel column and eluted with ethyl acetate and methyl

alcohol in different ratio. The eluants from EtOAc : MeOH (6 : 4) and (3 : 7) yielded compound AP-1 and AP-2 respectively which were purified as homogenous mass by repeated column chromatography on Si gel seperately and preparative layer chromatography by using solvent system (Benzene : Pyridine : Formic acid, 36 : 9 : 5) [Kamil *et. al.*, 1987]

STUDY OF COMPOUND AP – 1 :-

Compound AP-1, light brown amorphous solid m.p. 204 - 5^{0}C. It gave pink colour with shinoda test for flavonoids [Geissman, 1955]. Molecular formula $C_{44}H_{30}O_{13}$ as calculated from FABMS molecular ion peak $[M + H]^+$ 767.

PRESENCE OF IMPORTANT FUNCTIONAL GROUP IN AP-1 :-

The IR spectrum of AP-1 confirmed the important structural unit as hydroxy band at 3462 cm^{-1} , hydrogen bonded carbonyl band at 1652 cm^{-1} and gem dimethyl grouping at 1360 cm^{-1} on the basis of available literature [Nakanishi, 1962, Silverstein *et.al.*, 1974].

IR chart

UV SPECTRUM OF AP-1 :-

The UV spectrum of AP-1 displayed absorption maxima with various shift reagents as follow -

λ(MeOH)max	256	261	348	
log ε	4.20	4.13	4.4	
λ (+NaOMe)max	272	398		
log ε	4.28	4.39		
λ (+$AlCl_3$)max	272	298	412	
log ε	4.72	3.91	4.15	
λ(+$AlCl_3$/HCl)max	261	274	292	380
log ε	4.31	4.32	4.31	4.12
λ(+NaOAc)max	265	415		
log ε	4.21	4.20		
λ(+NaOAc/H_3BO_3)max	258	376		
	4.21	4.37		

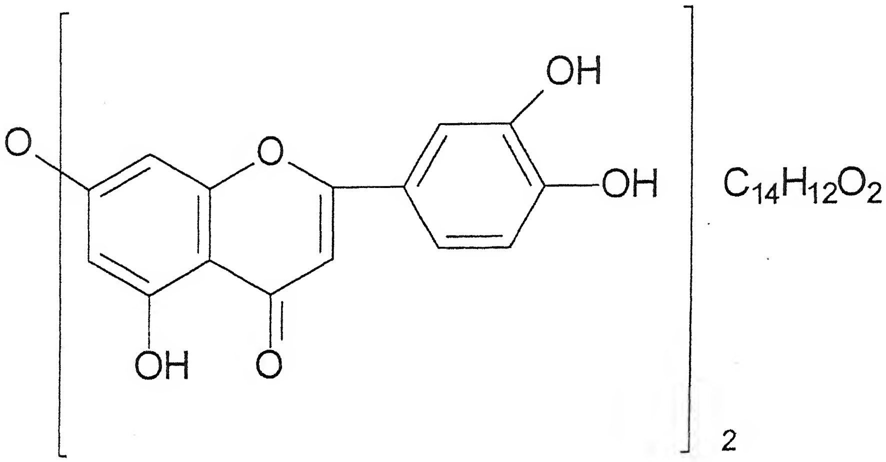
The spectrum in methanol exhibited characteristic bands similar to luteolin [Greenham *et.al.*, 2001]. It was supported by the bathochromic shift of 28 nm in band I in the presence of NaOAc/H_3BO_3 relative to MeOH and 32 nm shift with $AlCl_3$ relative to $AlCl_3$/HCl suggested the presence of *ortho* dihydroxy nature of ring B. There was no pronounced shift observed with NaOAc relative to MeOH led to conclude the absence of free OH at C-7 position [Mabry *et.al.*,1970]. The UV spectrum showed bathochromic shift of 32 nm in band I a on addition of $AlCl_3$/HCl suggesting hydroxyl at C-5 [Mabry *et.al.*, 1970].

BIFLAVONOID NATURE :-

The observance of high molecular ion peak $(M+H)^+$ at 767 indicated it to be a dimeric or biflavonoid structure. Though molecular extinction coefficient were not coinciding with that at typical biflavonoid molecule [Jackson *et.al.*, 1971]. However the etheral linkage between two molecule could be assumed

which was confirmed later on by FABMS fragmentation. [Jayaprakasam *et.al.*, 2000, Likhitwitayawuid *et.al.*, 2001].

On the basis of UV spectral and IR spectrum data a tentative structure of compound AP-1 could be assigned as (I)

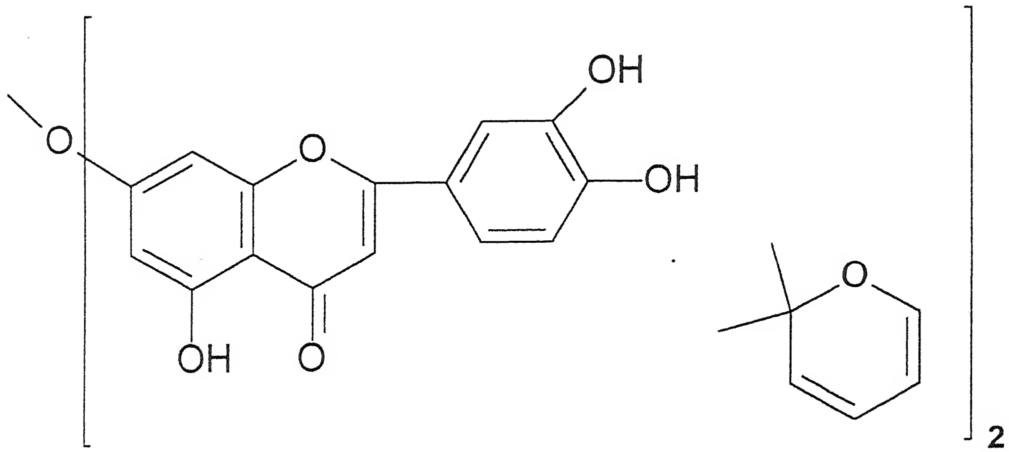


I

PRESENCE OF CHROMENE MOIETY :-

Gem dimethyl grouping adjacent to oxygen with olefins protons were attributed by the chemical shift at δ_H 1.21 (6H for 2 Me), δ_H 1.41 (6H for 2 Me) and δ_H 7.45 (2H, d, *J*=8.4 Hz) and 6.94 (2H, d, *J*=8.4 Hz) [Pathak *et.al.*, 1983]. The presence of chromene moiety in molecule was further strengthen by ^{13}C NMR spectrum exhibiting one upfield signal in aliphatic region at δc 29.2 and one quaternary signal at δc 79.7 [Kaouadji *et.al.*, 1986]. This was unequivocally corroborated by 2D NMR spectrum from HMQC. There were three cross peaks with carbon atom at δc 29.2, δc 116.2 and δc119.2 each encounters only one cross peak as δc/δ_H 29.2/1.21 (C-7'',8''/H-7'', 8'') δc/δ_H 29.2/1.41, δc/δ_H 116.2/6.94 (C-3''/H-3'') 119.2/7.45 (C-4''/H-4''). Carbon at δc 79.7, δc 121.5,

δc 151.5 showed no cross peak attributed no attachment of hydrogen at 2",5",6" [Silverstein *et.al.*,1998] . Thus the molecule of AP-1 could be depicted as (II)



II

STUDY OF PROTONATED FABMS OF AP-1:-

The fragmentation pattern of AP-1 from its protonated FABMS has been displayed in Fig-1. The molecular ion peak at m/z 767 $[M+H]^+$corresponded to molecular formula $C_{44}H_{30}O_{13}$. The yield of fragment at m/z 709 was result scissoring of chromene ring [Waterman, 1987]. It was further supported by the formation of fragments m/z 201 from m/z 216, because of only the dimethyl chromene nucleus is liable to loose one of the geminal methyl group as a free radical [Reed,1963], suggesting the presence of chromene moiety on B ring of flavone, followed by removal of chromene moiety and producing molecular ion at m/z 392 and m/z 307 respectively. Consequently double RDA [Drewes *et.al.*, 1967] in parent molecule fragment m/z 287 corroborated the interflavonyl etheral linkage at C-7. Further the appearance of peak with 100% intensity at m/z 154 appeared from A ring and suggested presence of OH at C-5.

STYDY OF ^{1}H NMR OF AP-1 :–

The various chemical shift observed in ^{1}H NMR have been displayed in Table-1, Fig –2 on the basis of available literature [Bhacca and Williams, 1964]

Table – 1

S.No.	Chemical Shift	Pattern	*J* value Hz	No. of protons	Assignments
1.	6.66	s	-	2	H-3
2.	6.21	d	2	2	H-6
3.	6.49	d	2	2	H-8
4.	7.42	s	-	2	H-2’
5.	6.94	d	8.4	2	H-3’’
6.	7.45	d	8.4	2	H-4’’
7.	1.21	s	-	6	H-7’’ or 8’’
8.	1.41	s	-	6	H-7” or 8”
9.	8.1-9.3	Broad hump	-	4	OH-3’,4’.
10.	12.5	s	-	2	OH-5

The appearance of a distinct sharp singlet at δ_H 6.66 was accounted for two protons at H-3. Presence of two broad singlet at δ_H 6.2 and δ_H 6.49 each for two proton could be assigned for position H-6 and H-8 proton. The chemical shifts for chromene ring have already been discussed on page 32.

STUDY OF ^{13}C NMR SPECTRA OF AP-1 :–

The ^{13}C NMR spectrum of AP-1 in DMSO at 75 MHz displayed 17 signals for 44 carbon Fig-3 out of these 14 signals were closely resembled to that of luteolin.[Agarwal and Bansal, 1989]. The δ values are given in Table - 2.

Presence of highly downfield shift of quaternary carbon atom at δc161.6, δc157.5, δc146.0, δc150.1 assignable for C-5, C-7, C-3' , C-4' confirmed the presence of OH at these position [Akdemir, *et.al.*, 2001].

Apart from this it also displayed signals for chromene have already been describe on page 32.

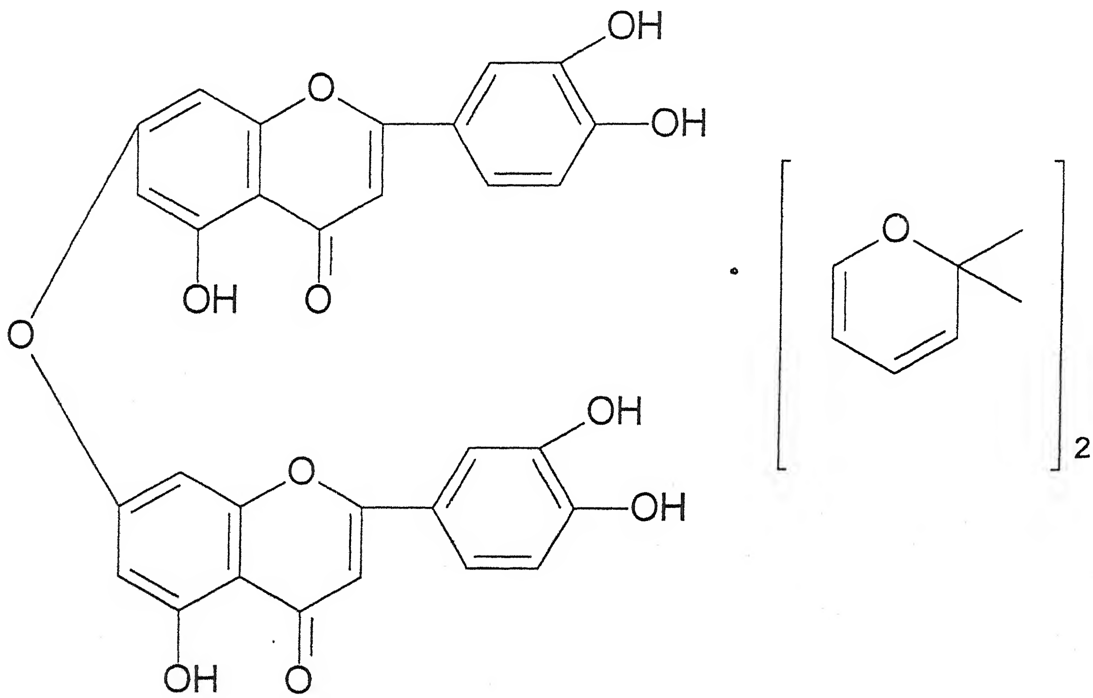
Table -2

S.N.	Chemical shift	Assignments	Luteolin.
1.	164.8	C-2	164.5
2.	103.7	C-3	103.3
3.	181.8	C-4	182.2
4.	161.6	C-5	162.1
5.	99.1	C-6	99.2
6.	157.5	C-7	164.7
7.	94.2	C-8	94.2
8.	157.5	C-9	157.9
9	103.7	C-10	104.2
10.	119.2	C-1'	119.3
11.	113.4	C-2'	113.8
12.	146.0	C-3'	146.2
13.	150.1	C-4'	150.1
14.	102.9	C-5'	116.4
15.	121.5	C-6'	122.1
16.	79.7	C-2''	
17.	116.2	C-3''	
18.	119.2	C-4''	
19.	121.5	C-5''	
20.	157.5	C-6''	
21.	29.2	C-7''	
22.	29.2	C-8''	

HMQC SPECTRUM OF AP-1 –:

The HMQC spectrum of AP-1 has been shown in Fig. 4. All the protons were clearly correlated with crosspeaks at δ_C 103.7/δ_H 6.66, δ_C 99.1/ δ_H 6.21, δ_C 94.2/δ_C 6.49 for C-3/H-3, C-6/H-6, and C-8/H-8 respectively and supported the presence of proton at C-3 , C-6 , & C-8 positions.

On the basis of above discussion the proposed structure for AP-1



(III)

ATTACHMENT OF CHROMENE TO FLAVONE IN AP-1 : -

The attachment of chromene to flavone nucleus could be assigned on the basis of following evidences.

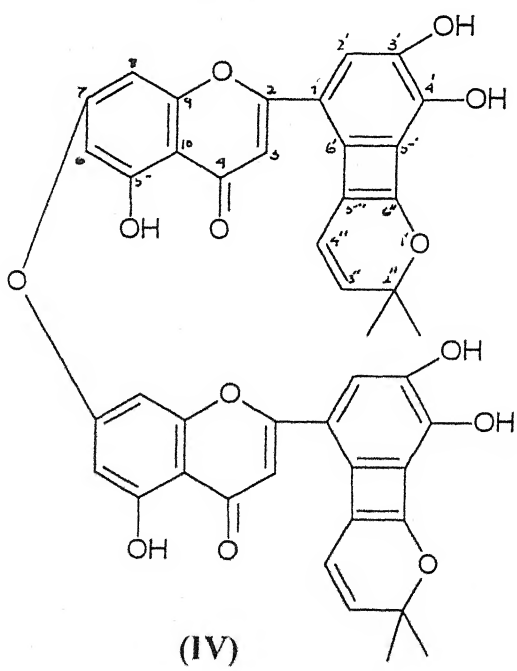
(1) Observation of fragment m/z 201 and m/z 216 were supportive of chromene ring on B ring.

(2) The *ortho* dihydroxy nature of B ring of flavonoidal unit as inferred due to observation of bathochromic shift in UV spectrum in presence of NaOAc/H_3BO_3 and $AlCl_3$.

(3) Availability of only one free proton on ring B beacause of the sharp singlet in up field region at δ_H 7.42 suggested chromene ring at 5' and 6' position.

(4) Quaternary signal for 5'6' and 5''6'' in ^{13}C NMR spectra were indicative of linear attachment of chromene ring.

(5) Therefore on the basis of above evidences it was concluded that the chromene ring was attached on B ring of flavonoidal skeleten on 5',6' position through C-C bonds forming cyclobutadine ring in molecule, and strain induced to its antiaromatic character was nullified perhaps due to bulky substituents on both ends providing stability. (March,1992). On the basis of aforesaid spectral evidences the molecular structure of AP-1 (IV) could be deduced as **Bis dimethyl chromene [5',6': 5'',6''] 7-0-7 biluteolin.**



(IV)

FAB MASS SPECTRUM OF COMPOUND - AP - 1

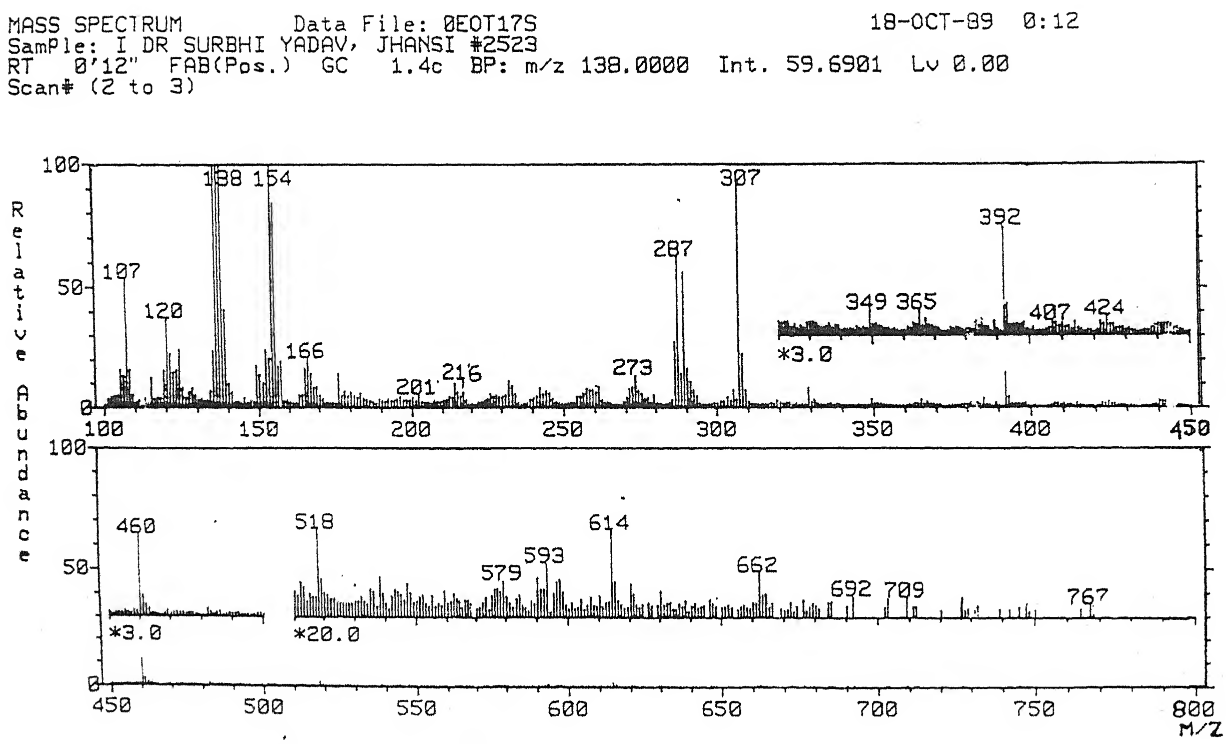


FIG - 1

^1H NMR SPECTRUM OF COMPOUND - AP - 1

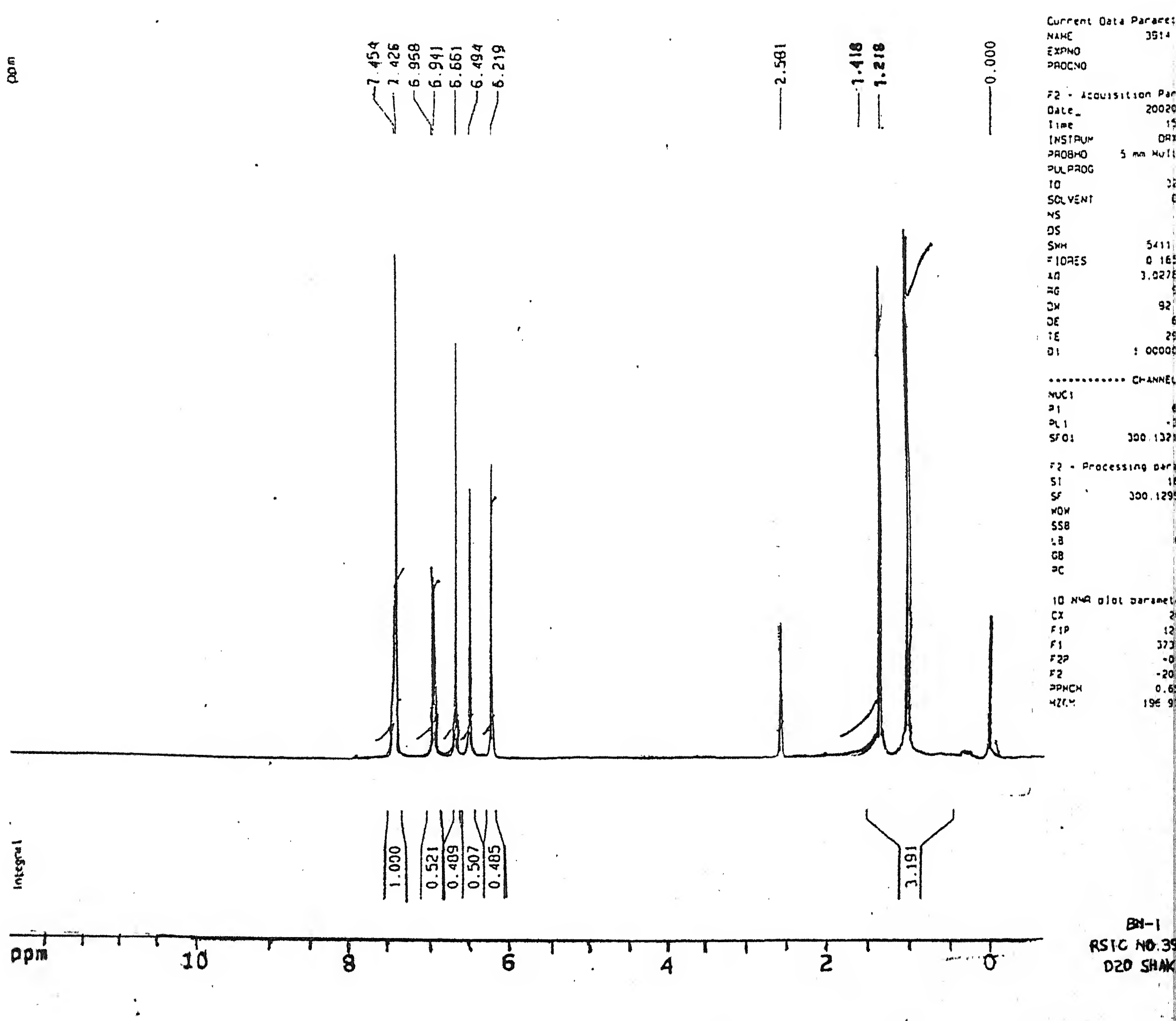


FIG - 2

^{13}C NMR SPECTRUM OF COMPOUND - AP - 1

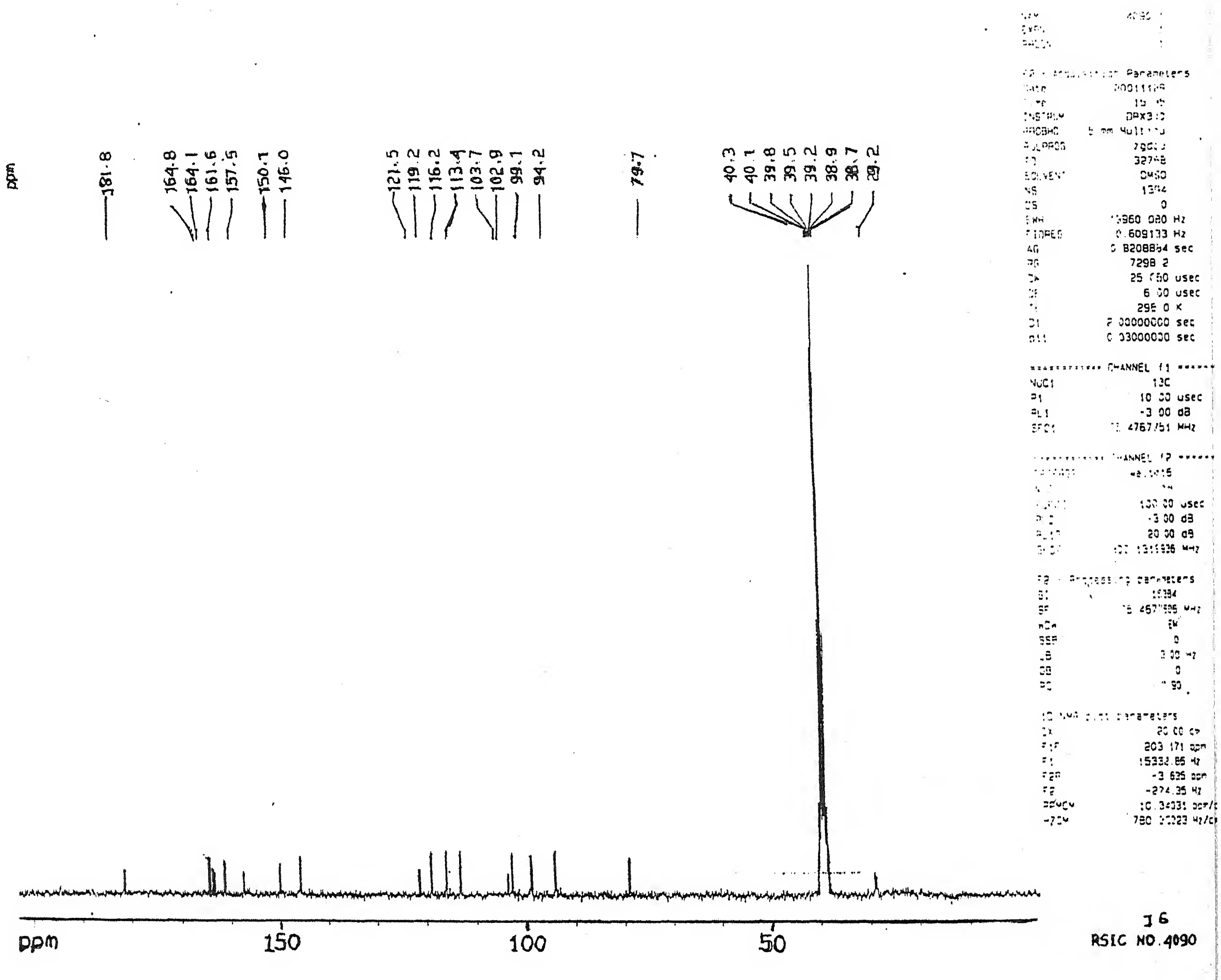


FIG - 3

HMQC SPECTRUM OF COMPOUND AP - 1

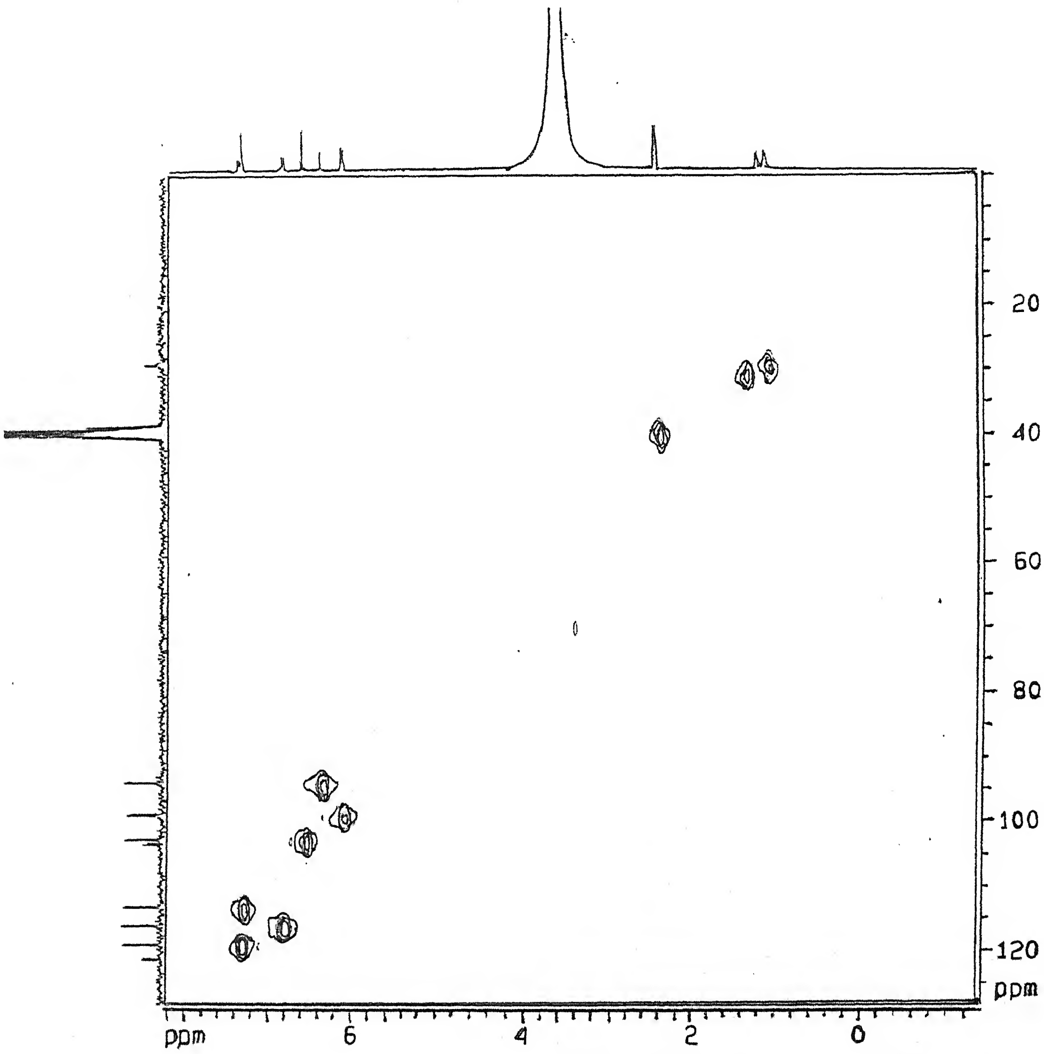
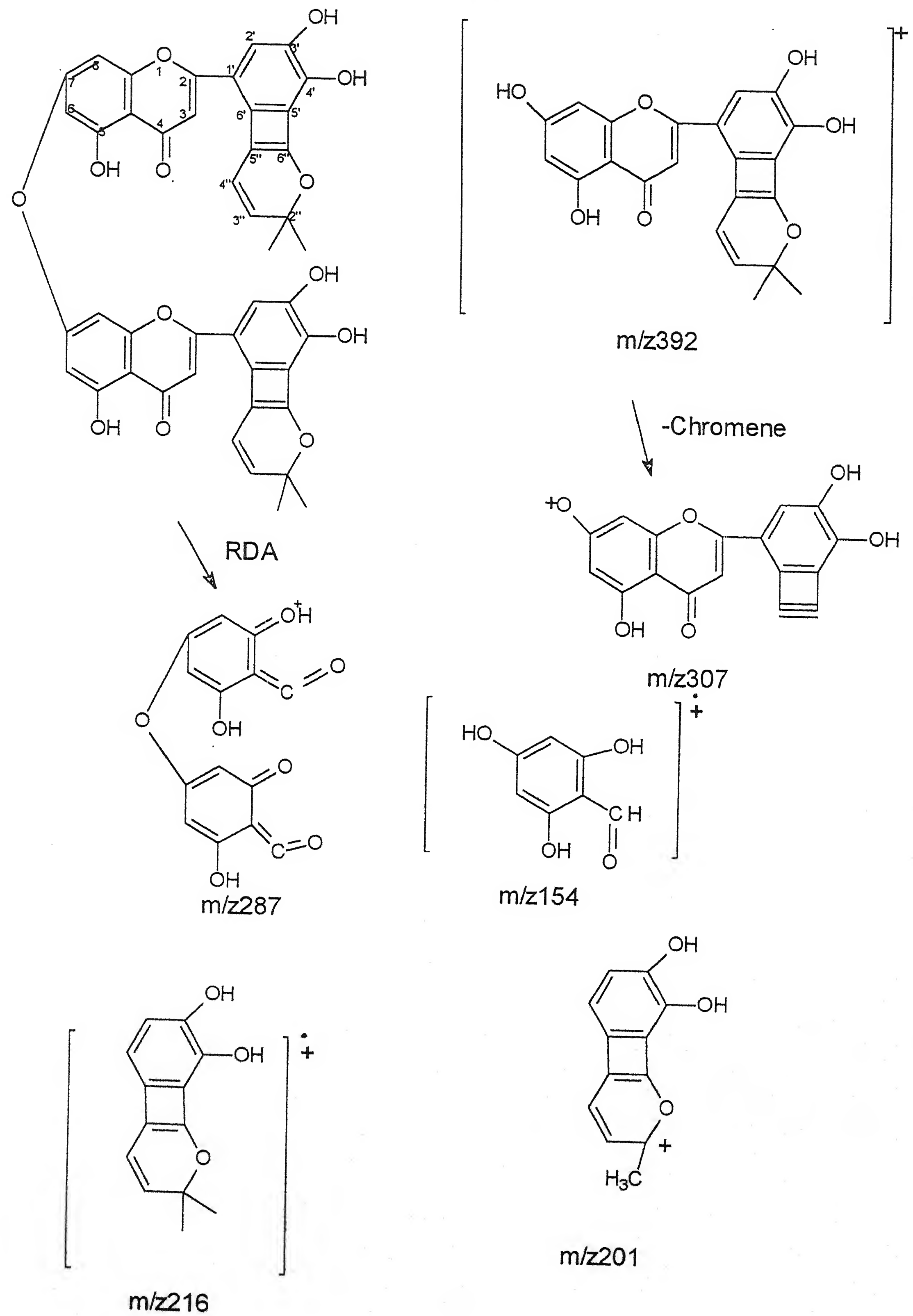


FIG - 4

SCHEME - 1



STUDY OF COMPOUND AP-2 :-

Compound AP-2 a yellow amorphous substance crystallised from ethanol m.p. 270 - 72^{0}C. The compound responded shinoda test [Geissman, 1955] and Borntrager reaction [Robinson, 1963] for flavonoid and anthraquinone respectively, its molecular formula $C_{44}H_{24}O_{16}$ due to $[M+H]^+$ at 809 in FABMS.

PRESENCE OF IMPORTANT FUNCTIONAL GROUP IN AP-2 :-

The recorded IR spectrum of AP-2 exhibited significant peaks at 3421 cm^{-1} for hydroxy group, 1658 cm^{-1} for chelated carbonyl group, 1620 cm^{-1} for α–hydroxy anthraquinone. The structural identity of functional groups were confirmed with available literature [Bellamy, 1958, Bloom *et.al.*, 1959].

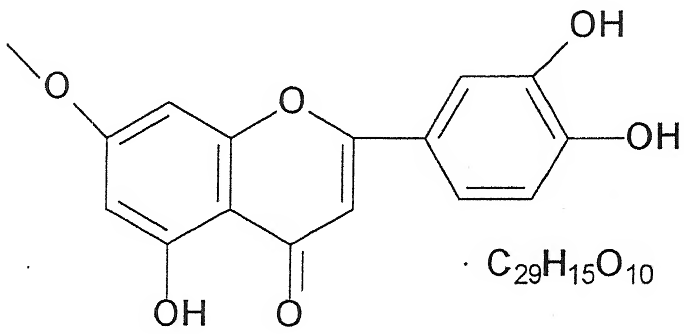
UV SPECTRUM OF AP – 2 :-

The UV spectrum of AP-2 exhibited absorption maxima with various shift reagents as-

λ (MeOH)$_{max}$	255	264	347	
log ε	4.32	4.21	4.54	
λ (+NaOMe)$_{max}$	263	402		
log ε	4.34	4.94		
λ (+$AlCl_3$)$_{max}$	270	302	324	
log ε	4.61	4.50	4.24	
λ (+$AlCl_3$/HCl)$_{max}$	273	293	361	380
log ε	4.39	4.34	4.21	4.38
λ (+NaOAc)$_{max}$	264	398		
log ε	4.71	4.42		
λ (+NaOAc/H_3BO_3)$_{max}$	255	366	374	
log ε	4.70	4.38	4.70	

The UV spectrum of AP-2 showed characteristic bands for flavone. It also showed resemblance with that of luteolin. [Greenham *et.al.*, 2001]. The pronounced bathochromic shift of 27 nm in band I was observed in spectrum with $NaOAc/H_3BO_3$ relative to MeOH suggested the presence of 3',4' *ortho* dihydroxy nature of B ring in flavonoid molecule. The addition of NaOAc in MeOH spectrum was displaced to 9nm (band II) relative to MeOH, indicated absence of free OH group at C-7 position [Mabry *et.al.*, 1970]. The spectrum of AP-2 on addition of $AlCl_3/HCl$ in MeOH exhibited four major bands all shifting bathochromically relative to MeOH indicating C-5 hydroxylation in flavone molecule. [Mabry *et.al.*, 1970].

Therefore on the basis of UV data a tentative structure for AP-2 could be assumed as V.



$C_{29}H_{15}O_{10}$

(V)

ACID HYDROLYSIS OF AP-2 :-

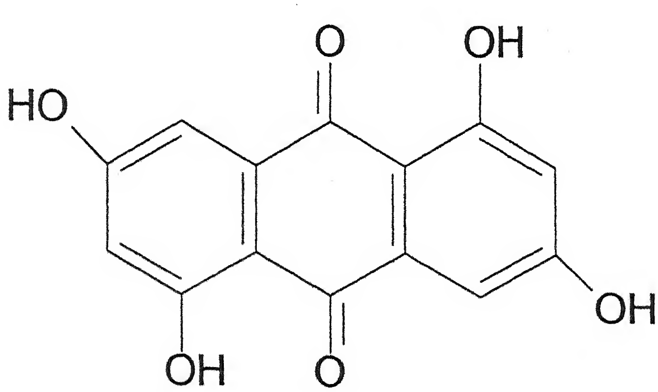
On acid hydrolysis with 7% H_2SO_4 [Saxena and Shrivastav, 1986] the resultant hydrolysate showed two spots on TLC on silica gel G, which were separated by PLC using $CHCl_3$: MeOH (9 : 1) solvent system . The separated bands were designated as AP-2' and AP–2''.

STUDY OF AP-2' :-

AP-2' was an orange colour microcrystalline substance m.p. 268-70^{0}C answering characteristic reaction for anthraquinone [Robinson, 1963. Thomson, 1971]. It was soluble in Na_2CO_3 solution which indicated presence of hydroxyl group on α – position of molecule [Briggs *et.al.*, 1957]. It gave orange red colour with 5% methanolic magnesium acetate suggestive of OH group at C-3 position [Shibata and Tanaka, 1950]. The exceptionally low frequency IR peak at 1610cm^{-1} in AP-2 molecule due to conjugated chelation and strong hydrogen bonding because of dipolar carbonyl group suggested presence of OH group adjacent to carbonyl group [Bloom *et.al.*, 1959]. The UV spectrum of AP-2' exhibited absorption maxima at λMeOH 256, 486 nm, the UV band at 486 nm suggested the absence of α – OH either on 1,4 or on 5,8 [Brockman and Miller, 1959]

In the ^{1}H NMR spectrum of AP-2 presence of doublet at δ_H 7.09 [2H, d, J= 2.4 Hz] and δ_H 6.98 [2H, d, J = 2.4 Hz] could be assigned 2"6" and 4", 8" [Alemayehu *et.al.*, 1996]

Thus on the basis of UV, ^{1}H NMR and complementary support of chemical test the structure of AP-2' could be assigned as 1,3,5,7 tetrahydroxy anthraquinone. VI.



VI

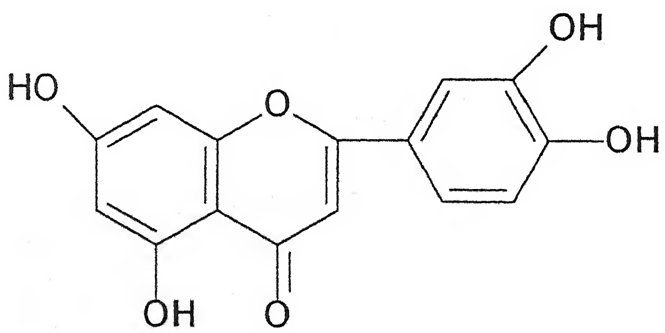
STUDY OF AP-2" :-

Compound AP-2" was cream colour substance m.p. 258-60^0 showed cherry red colour with Mg/HCl. The substance AP-2" was characterized as luteolin 5,7,3',4' tetra hydroxy flavone (VII) by mixed m.p. and co-chromatography with authentic specimen. Rf 0.78 (TBA).

UV SPECTRUM OF AP-2" :-

The UV spectrum of AP-2" exhibited absorption maxima with various shift reagents as

$\lambda\ (MeOH)_{max}$	258, 267, 348
$\lambda\ (+NaOMe)_{max}$	329, 402
$\lambda\ (+AlCl_3)_{max}$	274, 300, 328, 426
$\lambda\ (+AlCl_3/HCl)_{max}$	266, 275, 355, 388
$\lambda\ (+NaOAc)_{max}$	267, 327 sh, 385
$\lambda\ (+NaOAc/H_3BO_3)_{max}$	259, 302, 370, 428



(VII)

STUDY OF ^{1}H NMR OF AP-2 :-

The assignment of various chemical shift of protons present in AP-2 have beeen made with earlier reported literature [Wibery and Nist, 1962] and arranged in Table – 3. Fig – 5

The ^{1}H NMR of AP-2 in DMSO– d_6 using TMS as internal standard exhibited chemical shifts accounting for 24 protons of two flavonoid units, with anthraquinone. Presence of chemical shift as sharp singlet at δ_H 6.57 corresponding to two protons was due to location on H-3 [Maatooq *et.al.*, 1997]. The broad hump appeared at δ_H 8.7 to δ_H 9.4 integrated for eight protons corroborated the presence of eight hydroxyl protons as suggested by UV spectrum.

Table – 3

S.No.	Chemical shift	Pattern	*J* value Hz	No. of protons	Assignment
1.	6.57	s	-	2	H-3
2.	6.24	d	2	2	H-6
3.	6.48	d	2	2	H-8
4.	7.57	d	1.2	4	H-2',6'
5.	7.40	d	1.2	2	H-5'
6.	7.09	d	2.4	1	H-2''
7.	6.98	d	2.4	1	H-4''
8.	7.09	d	2.4	1	H-6''
9.	6.98	d	2.4	1	H-8''
10.	8.7 to 9.4	broad hump	-	8	OH-3'4' 1'' 3'' 5'' 7''
11.	13.4	s	-	2	OH-5

The presence of proton signals located at δ_H 7.40 [d, J = 1.2 Hz] and δ_H 7.57 [d, J=1.2Hz] for *meta* coupled proton representing 6 protons of C-2',6' and 5' in the B ring of flavonoids. The four *meta* coupled protons at δ_H 6.24 (2H, d, J=2 Hz) and δ_H 6.48 (2H, d, J = 2 H) appeared for A ring protons at H-6 and H-8. The chemical shifts inferred for protons of anthraquinone are discussed on prerequisite place on page 41.

STUDY OF ^{13}C NMR OF AP-2 :-

The signals obtained from ^{13}C NMR of AP-2 in DMSO-d_6 have been tabulated in Table-4 Fig-6

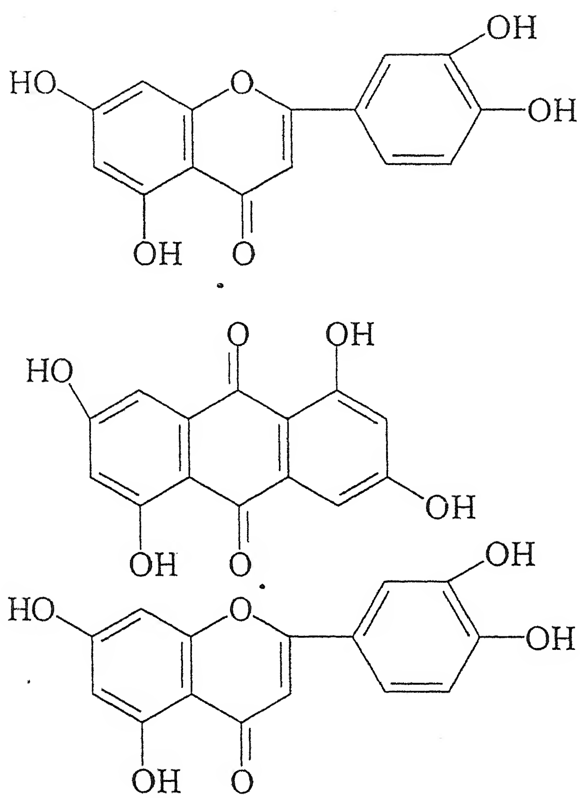
There were 22 signals in aromatic region were observed corresponding to 44 carbon for two flavonoid unit in molecule due to C_2 symmetry of molecule [Hamzah *et.al.*,1997] Out of these δ_C165.2, δ_C104.3, δ_C183.1, δ_C163.4, δ_C99.8, δ_C165, δ_C94.8, δ_C158.9, δ_C105.4 ; δ_C120.2, δ_C114.2, δ_C146.6, δ_C150.2, δ_C116.7, δ_C123.8, were coincide with that of luteolin reported earlier [Agarwal and Bansal, 1989]. Further the presence of chemical shift in high upfield region at δ_C 99.6 [C-6] and δ_C 99.8 [H-8] corroborated the unsubstituted C-6 and C-8 position [Likhitwitayawuid *et.al.*, 2001].

Table – 4

S.No.	Chemical Shift	Assignments	Luteolin
1.	165.2	C-2	164.5
2.	104.3	C-3	103.3
3.	183.1	C-4	182.2
4.	163.4	C-5	162.1
5.	99.8	C-6	99.2
6.	165.0	C-7	164.7
7.	94.8	C-8	94.2
8.	158.9	C-9	157.9
9.	105.4	C-10	104.2
10.	120.2	C-1'	119.3
11.	114.2	C-2'	113.8
12.	146.6	C-3'	146.2
13.	150.2	C-4'	150.1
14.	116.7	C-5'	116.4
15.	123.8	C-6'	122.1
16.	163.4	C-1''	
17.	129.3	C-2'	
18.	152.6	C-3''	
19.	123.5	C-4''	
20.	163.4	C-5''	
21.	129.3	C-6''	
22.	152.6	C-7''	
23.	123.5	C-8''	
24.	184.2	C-9''	
25.	184.2	C-10''	

Where as seven chemical shifts comprising of 14 carbon atom in low field at δ_C165.2, δ_C163.4, δ_C165.0, δ_C146.6, δ_C150.2, δ_C152.6, δ_C163.4, were observed due to presence of OH at these position [Yahara *et.al.*, 2000].

On the basis of above discussion it was concluded that the AP-2 molecule was composed of two luteolin with one anthraquinone having a symmetric structure VIII..



(VIII)

STUDY OF PROTONATED FAB MASS SPECTRA OF AP-2 :-

The various fragment ion peaks observed in the FABMS were at,809$[M+H]^+$,m/z 676, 392,287,157,137,107, shown in scheme –2, Fig-7.

The $[M+H]^+$ at 809 suggested molecular formula $C_{44}H_{24}O_{16}$. The Retro Diel's Alder cleavage at m/z 676 was supportive of linkage of A ring of luteolin to

anthraquinone moiety [Juma *et.al.*, 2001]. The molecular ion at m/z 392 and m/z 286 strongly indicated that the molecule was consisted of the two luteolin unit attached through anthraquinone. Presence of independent m/z at 107 corroborated the anthraquinone moiety. The appearance of fragment at m/z 137 and m/z 157 were resulted from A ring of flavone and anthraquinone respectively.

ASSIGNMENT OF POSITION OF LUTEOLIN ON ANTHRAQUINONE :-

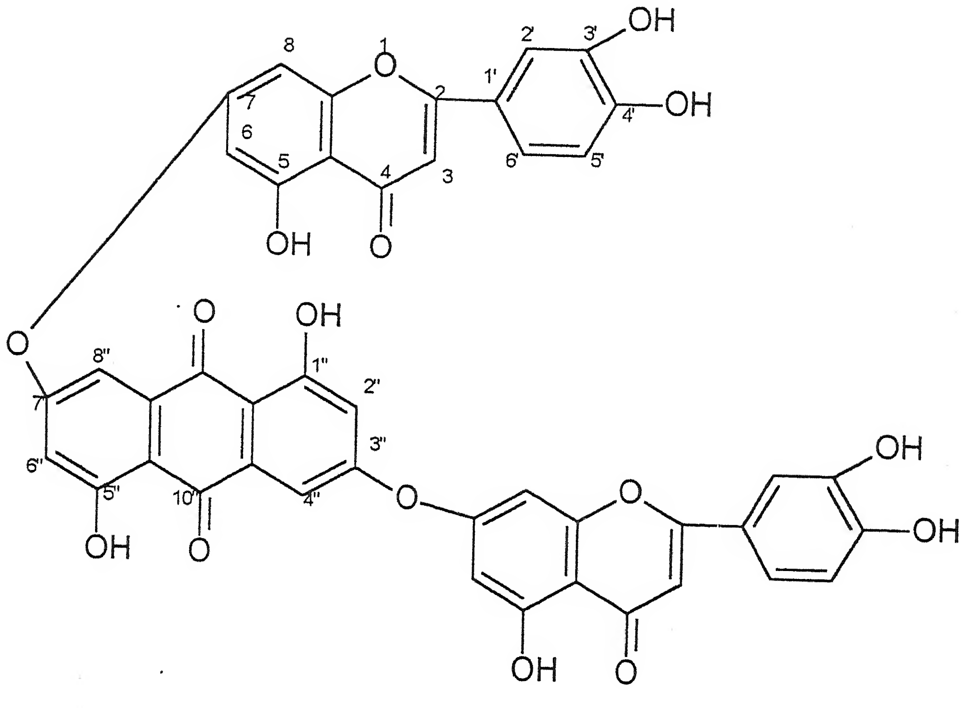
(a) 1,5 substitution in anthraquinone has already been inferred on the page no 41. Therefore the vacant position for linkage were either 2,3,4 or 6,7,8,.

(b) The presence of *meta* coupled proton was evident from the appearance of doublet at δ_H 7.09 (J=2.4 Hz) and δ_H at 6.98 (J=2.4 Hz) led to assume the 3^{rd} and 7^{th} position for option of attachment

(c) The presence of C_2 symmetry in molecule as confirmed by ^{13}C NMR data which corroborated the possibility of 3^{rd} and 7^{th} position for attachment of luteolin.

(d) AP–2 answered colour reaction with 5% magnesium acetate was indication of appearance of OH after cleavage of -*O*- linkage at 3''and 7'' position of anthraquinone.

All the evidences could be assembled to deduce the molecular structure of AP-2 as (7-3" : 7-7") **biluteolin 1",5" dihydroxy anthraquinone.** (IX).



(IX)

¹H NMR SPECTRUM OF COMPOUND - AP - 2

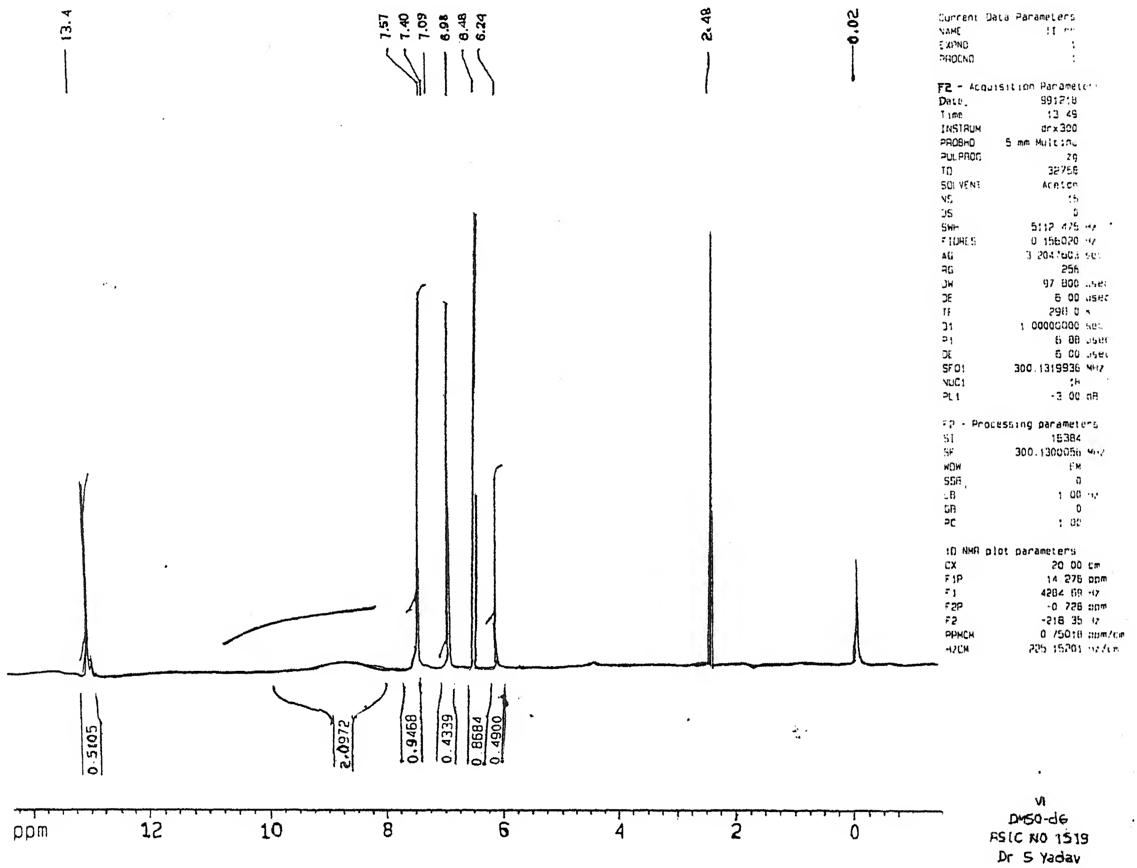
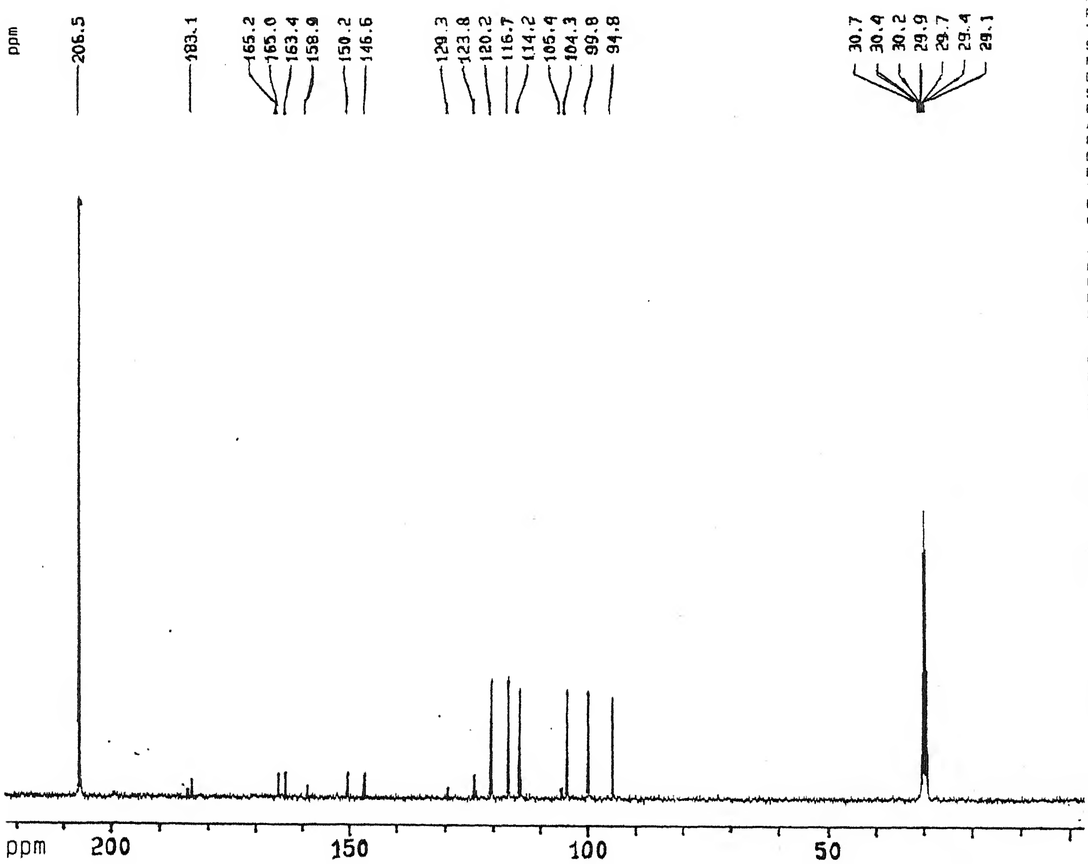


FIG - 5

^{13}C NMR SPECTRUM OF COMPOUND - AP - 2



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PROCNO	1

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FIDRES	0.609133 Hz
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RG	9195.2
DW	~25.050 use
DE	6.00 use
TE	298.0 K
D1	2.00000000 sec
d11	0.03000000 sec

============ CHANNEL f1 ===

NUC1	13C
P1	10.00 use
PL1	-3.00 dB
SFO1	75.4767751 MHz

============ CHANNEL f2 ===

CPDPRG2	waltz16
NUC2	1H
PCPD2	100.00 use
PL2	-3.00 dB
PL12	20.00 dB
SFO2	300.1319936 MHz

F2 - Processing parameters

SI	16384
SF	75.4676753 MHz
WDW	EM
SSB	0
LB	3.00 Hz
GB	0
PC	0.90

1D NMR plot parameters

CX	20.00 cm
F1P	222.338 ppm
F1	16779.33 Hz
F2P	-2.997 ppm
F2	-226.21 Hz
PPMCM	11.26677 ppm/
HZCM	850.27692 Hz/c

FIG - 6

FAB MASS SPECTRUM OF COMPOUND - AP - 2

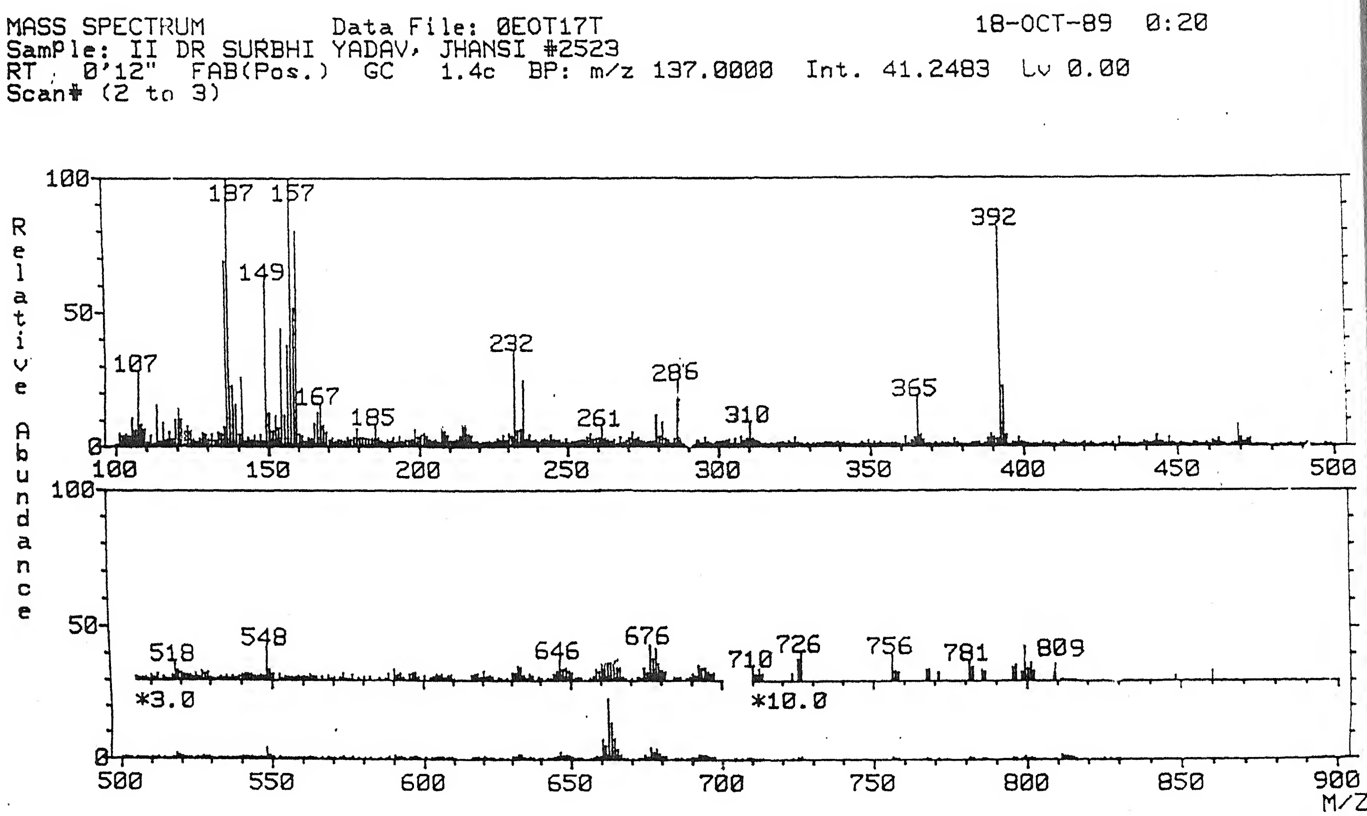
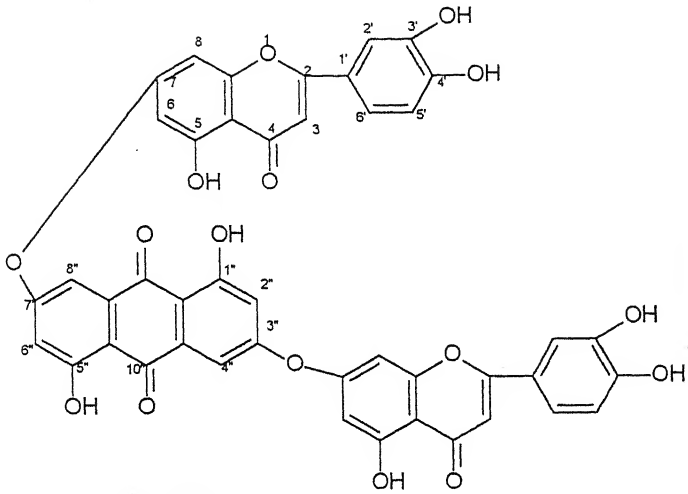
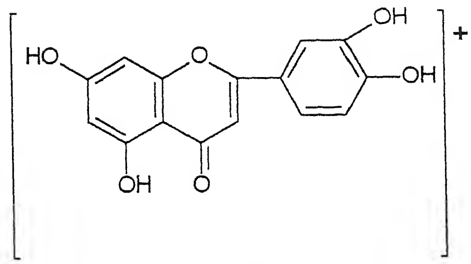


FIG - 7

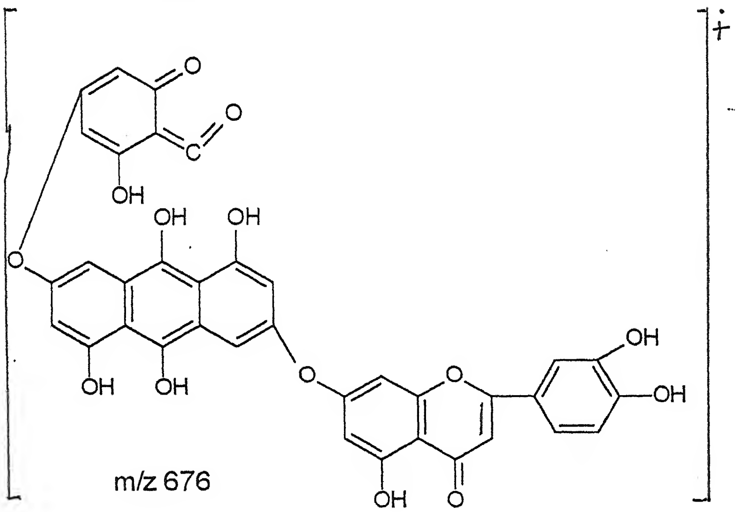
SCHEME - 2



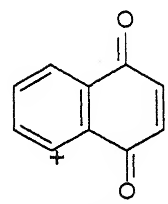
m/z 808



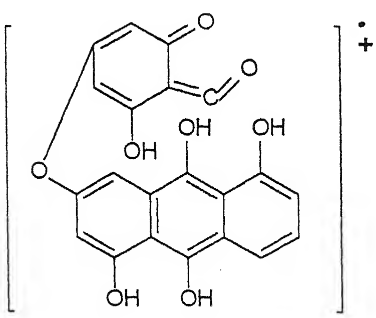
m/z 286



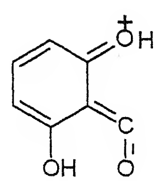
m/z 676



m/z 157



m/z 392



m/z 137

MASS SPECTRAL FRAGMENTATION OF AP-2

ISOLATION AND STUDY OF COMPOUND AP- 3 :-

Fresh leaves of *Albizia procera* were blended in mixer with acetone water (7:3) containing 0.1% ascorbic acid. The whole extract was filtered through a buchner funnel. The total aqueous acetone extract was reduced to fully aqueous phase under vacuum after removing organic solvent. The resulting aqueous phase successively extracted with ethyl ether and ethyl acetate and these were discarded. The aqueous phase diluted to 50% methanol and was charged over Sephadex LH-20 column and eluted with different fraction of water : methanol. The collected eluant from 50% methanol were lyophilized as brown solid which was purified by preparative TLC on cellulose using BAW solvent system to yield compound AP-3 (Rf = 0.85).

STUDY OF COMPOUND AP-3 :-

Compound AP-3 was dark yellow solid answered dark pink colour with Mg/HCl for flavonoid [Geissman, 1955], melted at 216-18^{0}C. Protonated FABMS $[M+H]^+$ 811 suggested molecular formula $C_{44}H_{26}O_{16}$.

PRESENCE OF IMPORTANT FUNCTIONAL GROUP IN AP-3 :-

The observed IR peaks of AP-3 were assigned to functional identities on the basis of available literature [Silverstein *et.al.*, 1974, Conley, 1972] . The prominent peaks as broad band at 3419 cm^{-1} and sharp band at 1656 cm^{-1} were ascribed to OH and chelated carbonyl group.

UV SPECTRUM OF AP-3 :-

The AP-3 displayed absorption with various shift reagents were as follow –

1.	λ (MeOH) $_{max}$	258	270	348		
	log ε	6.39	6.3	6.45		
2.	λ (+NaOMe) $_{max}$	275	402			
	log ε	6.32	6.47			
3	λ (+$AlCl_3$) $_{max}$	272	300	416		
	log ε	6.21	6.06	6.47		
4.	λ (+$AlCl_3$/HCl) $_{max}$	262	276	294	360	382
	log ε	6.36	6.36	6.1	6.2	6.45
5.	λ (+NaOAc) $_{max}$	268	414			
	log ε	6.32	6.32			

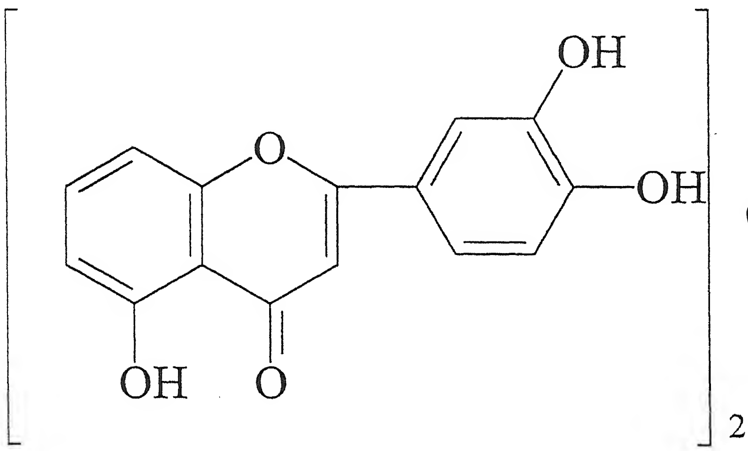
The bifurcation in band II at λ 258 and 270 nm in MeOH spectra indicated 3'4' hydroxylation inflavonoid moiety [Mabry *et.al.*, 1970] which was again supported by 34 nm hypsochromic shift on band I with $AlCl_3$/HCl relative to $AlCl_3$ characteristic for *ortho* dihydroxylation. [Mabry *et.al.*, 1970]. The displacement of all bands bathochromically in presence of $AlCl_3$/HCl relative to MeOH suggesting C-5 hydroxylation [Mabry *et.al.*, 1970].

BIFLAVONOIND NATURE OF AP-3 :-

Perusual of UV spectrum of AP-3 exhibited the resemblance to luteolin, but the absorption maxima did not correspond precisely with those calculated for two

moles of the monomers which indicated weak π−π. interaction between the two flavone unit [Jackson *et.al.*, 1973] and suggested biflavonoid nature of AP-3 which is again corroborated by high molecular ion peaks at $[M+H]^+$ 811 corresponding to molecular formula $C_{44}H_{26}O_{16}$.

On the basis of UV and IR spectra the tentative structure of AP-3 could be assumed as follows. (X)

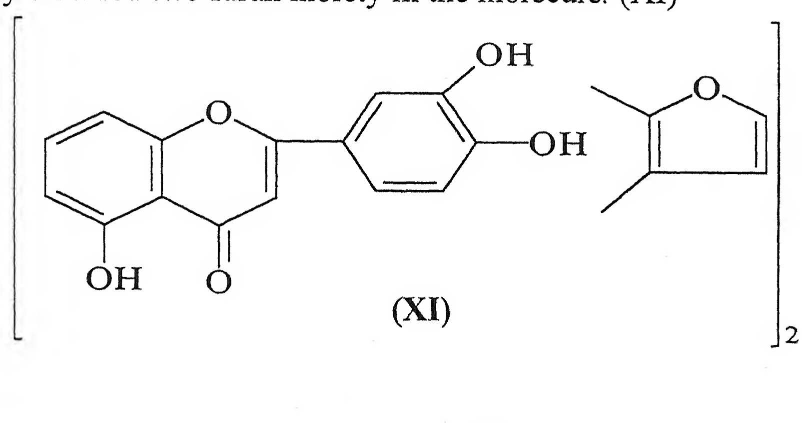


$C_{14}H_8O_6$

(X)

PRESENCE OF FURAN RING:-

In the IR spectrum of AP-3, presence of peaks at 1163 and 1569 cm^{-1} suggested the furan moiety [Finar, 1983] which was further corroborated by the observation of chemical shift at δ_H 6.24 (2H,d, *J*=1.2Hz) and δ_H 6.5 (2H, d *J*=1.2 Hz) in ^1H NMR spectra of AP-3 [Tanaka *et.al.*, 1992] suggestive of presence of equally shielded two furan moiety in the molecule. (XI)

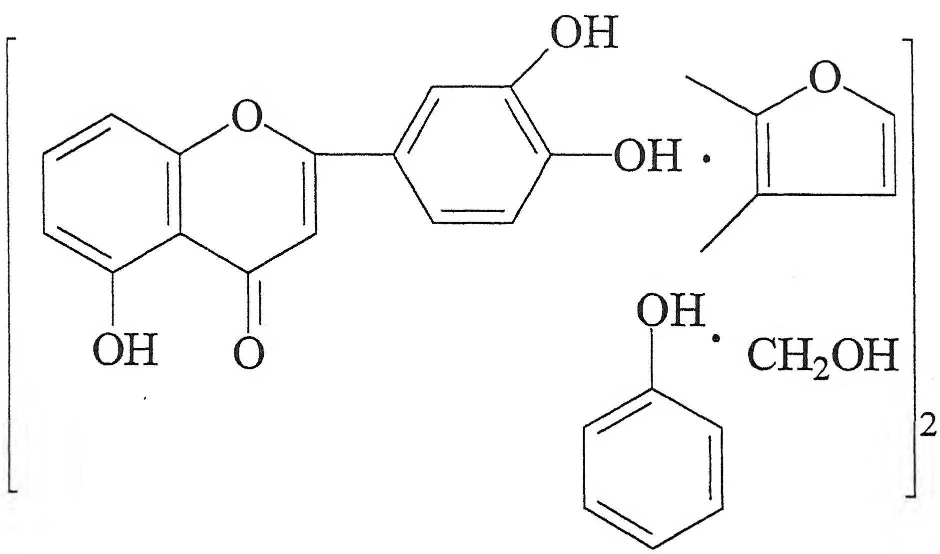


$C_{10}H_4O_4$

(XI)

PRESENCE OF PHENYL RING (D) AND ALIPHATIC ALCOHAL :-

Apart from two 5,3',4' trihydroxy flavone unit and two furan units in molecule. The remaining $C_{10}O_4H_4$ part could be ascribed to hydroxyl substituted phenyl moieties and primary alcohol grouping. These were inferred by observation of additional resonance in aromatic region. Signals of ortho and meta coupled protons at δ_H 7.4 (2H,d J=8.4Hz) and δ_H 6.98 (2H,d J=8.4Hz) and δ_H 7.49 (2H) at H-11,H-12,H-14 protons. Appearance of singlet at δ_H 2.04 and a multiplet at δ_H 3.70 led to conclude the presence of CH_2OH grouping at C-2' position.Further more in FABMS the yield of m/z 287 was resulted due to fused phenyl (D ring) with flavonoidal A ring . XII



(XII)

STUDY OF ^{1}H NMR of AP-3 :-

The ^{1}H NMR of AP-3 in DMSO-d_6 at 300 MHz exhibited the resonance for 26 protons (Table – 5, Fig -8).

Table –5

S.No.	Chemical Shift	Pattern	*J* value Hz	No. of Protons	Assignment
1	6.57	s	-	2	H-3
2-	7.49	s	-	2	H-14
3	7.4	d	8.4	2	H-11
4	6.98	d	8.4	2	H-12
5	3.70	m	-	4	H-1'''
6	6.24	d	1.2	2	H-4''
7	6.51	d	1.2	2	H-5''
8	13.01	s	-	2	OH-5
9	8.2-9.7	Broad hump	-	6	OH - 3', 4', 13
10	2.04	s	-	2	OH – 1'''

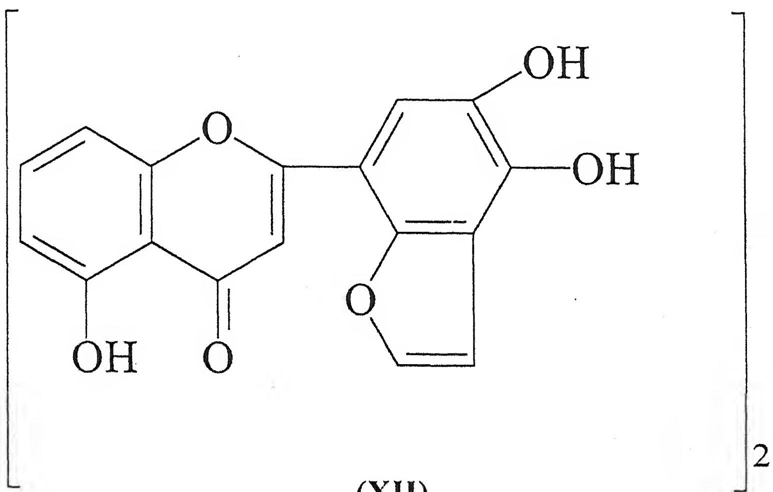
The location of a highly shielded chemical shift as singlet at δ_H 13.01 integrating for two chelated protons at C-5 [Tschan *et.al.*,1996] and a broad hump from δ_H 8.2–9.7 (D_2O exchangeable) corresponding for six proton of OH at C-3' and C-4' on both unit of flavone and remaining two protons could be assigned for OH group at D ring. The observation of singlet at δ_H 6.57 could be ascribed for two protons located at H-3 of I and II unit.

PROTONATED FAB MASS SPECTRA OF AP-3 :-

Positive FABMS exhibit fragmentation pattern with and without hydrogen transfer has been shown in scheme-3, Fig –9. The elemination of furan with CH_2 produced m/z 756. Odd electron species at m/z 662 were obtained by the breaking of furan and CH_2OH moiety with hydrogen transfer [Birch *et.al.*, 1967]. The peak at m/z 548 representing RDA with the removal of furan moiety [Mossa,1992] was suggestive interflavonyl linkage between A rings, further elimination of CHOH by loss of 30 mass unit gave molecular ion at m/z 518. The fragment at m/z 149 and m/z 107 were obtained from B ring and fragment 157 and 167 from A ring [Tanaka *et.al.*, 1992].

LINKAGE OF FURAN :-

The fragments m/z 149 and 107 in FABMS suggested the attachment of furan moiety on B ring. The presence of 3',4' hydroxylation on B ring of compound AP-3 has already been discussed by the UV spectral data on page 50 and corroborated by the D_2O exchangeable broad hump, left the possibility for furan at 5'6' of B ring (XII).

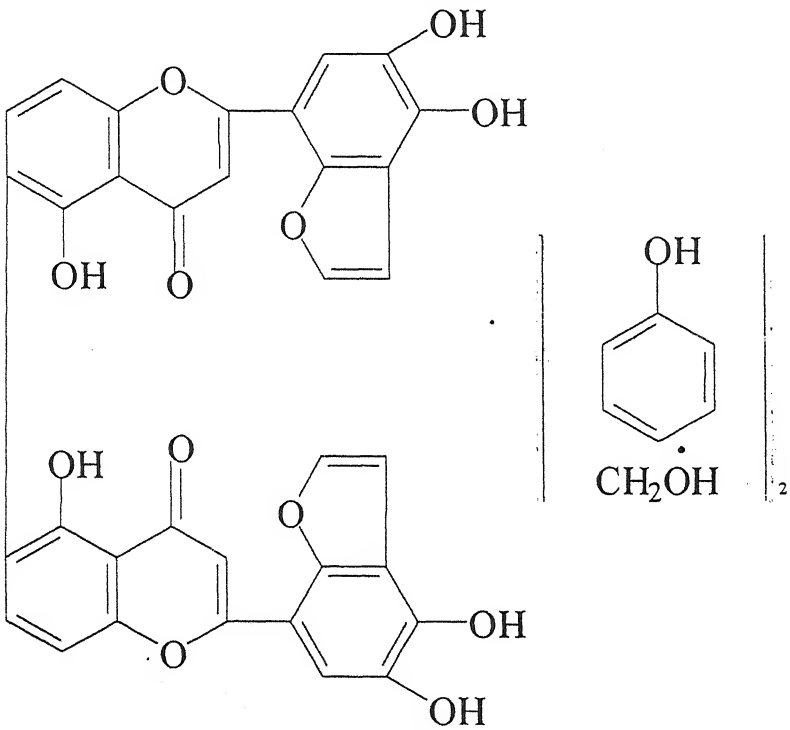


$C_{10}H_8O_4$

(XII)

INTERFLAVONOID LINKAGE :-

The fragment obtained from FABMS at m/z 662, 157 were suggestive of the linkage through A ring. However the earlier reports have envisaged that the linkage is always *ortho* to a hydroxy group and radical substitution reaction believed to be involved in *vivo* genesis of biflavone [Jackson *et.al.*, 1971] Thus the inter flavonyl linkage in AP-3 was assigned as I-C-6 to II-C-6 (XIV).



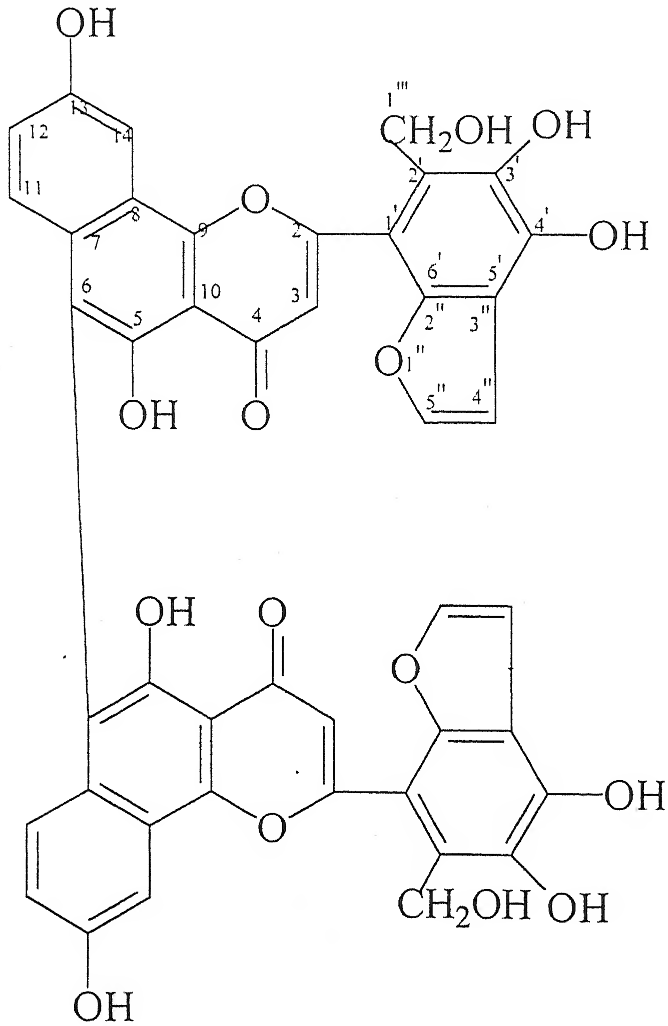
(XIV)

LINKAGE OF PHENOLIC RING (D) AND CH_2OH MOIETY :-

The perusal of FABMS pathway led to decide the fusion of phenolic ring on A ring of flavone, because of the yield of fragment m/z 548, 518, 167 [Roy *et.al.*, 1987]. Since the C-5 and C-6 position of A ring have already been allocated for OH and interflavonyl bond respectively leaving 7^{th} and 8^{th} position for phenolic ring. The presence of fragment at m/z 203 suggested CH_2OH moiety was

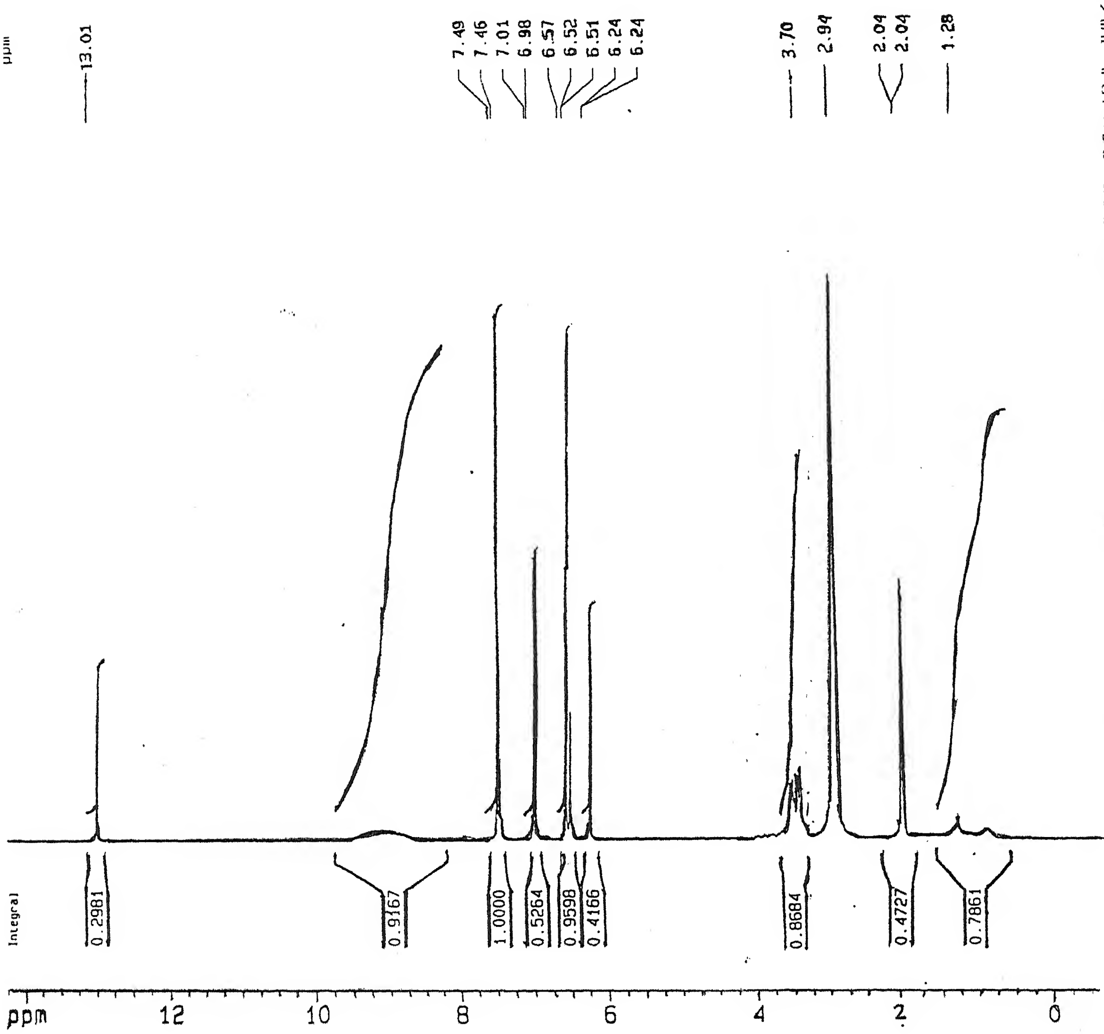
attached at B ring , on 2' position because 3'4' position has already being alloted for OH group and 5',6' position for furan, leaving only 2' position .

On accumulating all the evidences the structure of compound AP-3 could be deduced as **I-3', II-3' I-4' II-4' I-5 II-5 I-13 II-13 octahydroxy, I, II [2"3": 5',6'] difurano I-2', II-2'' bis hydroxy methyl I, II bis hydroxy phenyl [7, 8 : 15,16] I-6, II-6 biflavonoid (XV).**



(XV)

^{1}H NMR SPECTRUM OF COMPOUND - AP - 3



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RG 256
DW 97.800 usec
DE 6.00 usec
TE 298.0 K
D1 1.00000000 sec
P1 6.88 usec
DE 6.00 usec
SFO1 300.1319936 MHz
NUC1 1H
PL1 -3.00 dB

F2 - Processing parameters
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SSB 0
LB 1.00 Hz
GB 0
PC 1.00

1D NMR plot parameters
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F1P 14.276 ppm
F1 4284.69 Hz
F2P -0.728 ppm
F2 -218.35 Hz
PPMCM 0.75018 ppm/cm
HZCM 225.15201 Hz/cm

II
Acetone
RSIC NO. 1519
Dr. S. Yadav

FIG - 8

FAB MASS SPECTRUM OF COMPOUND - AP - 3

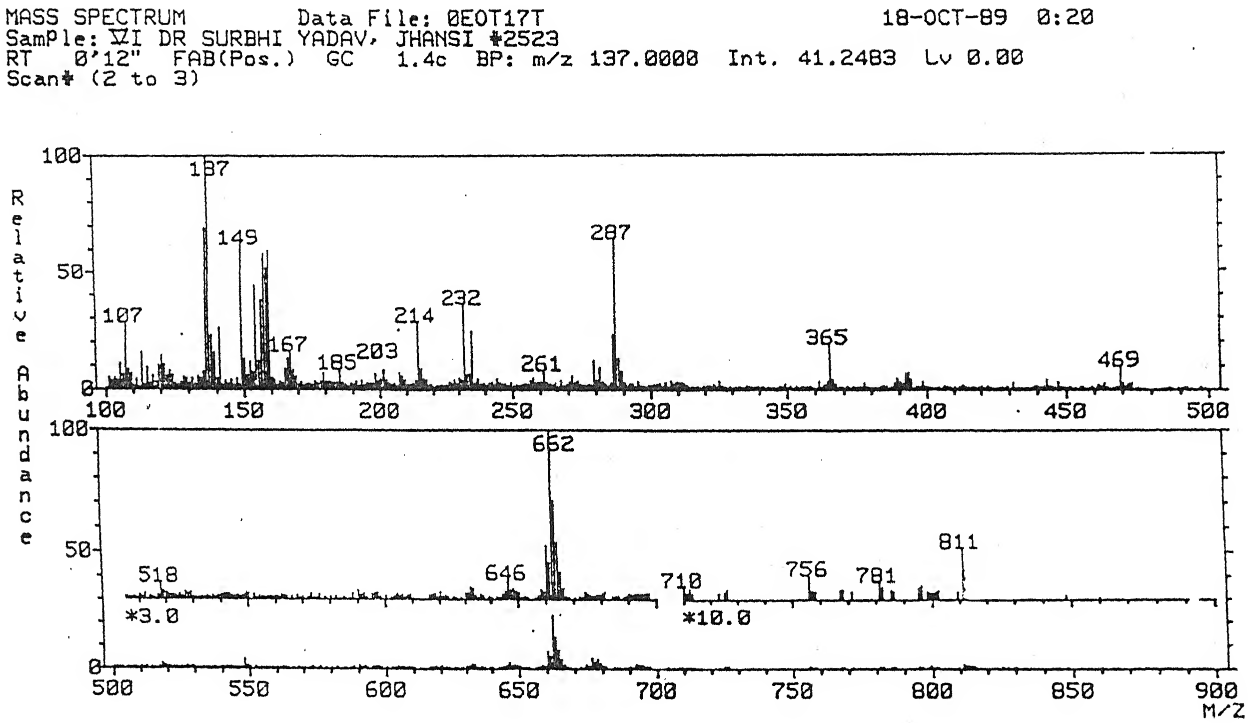
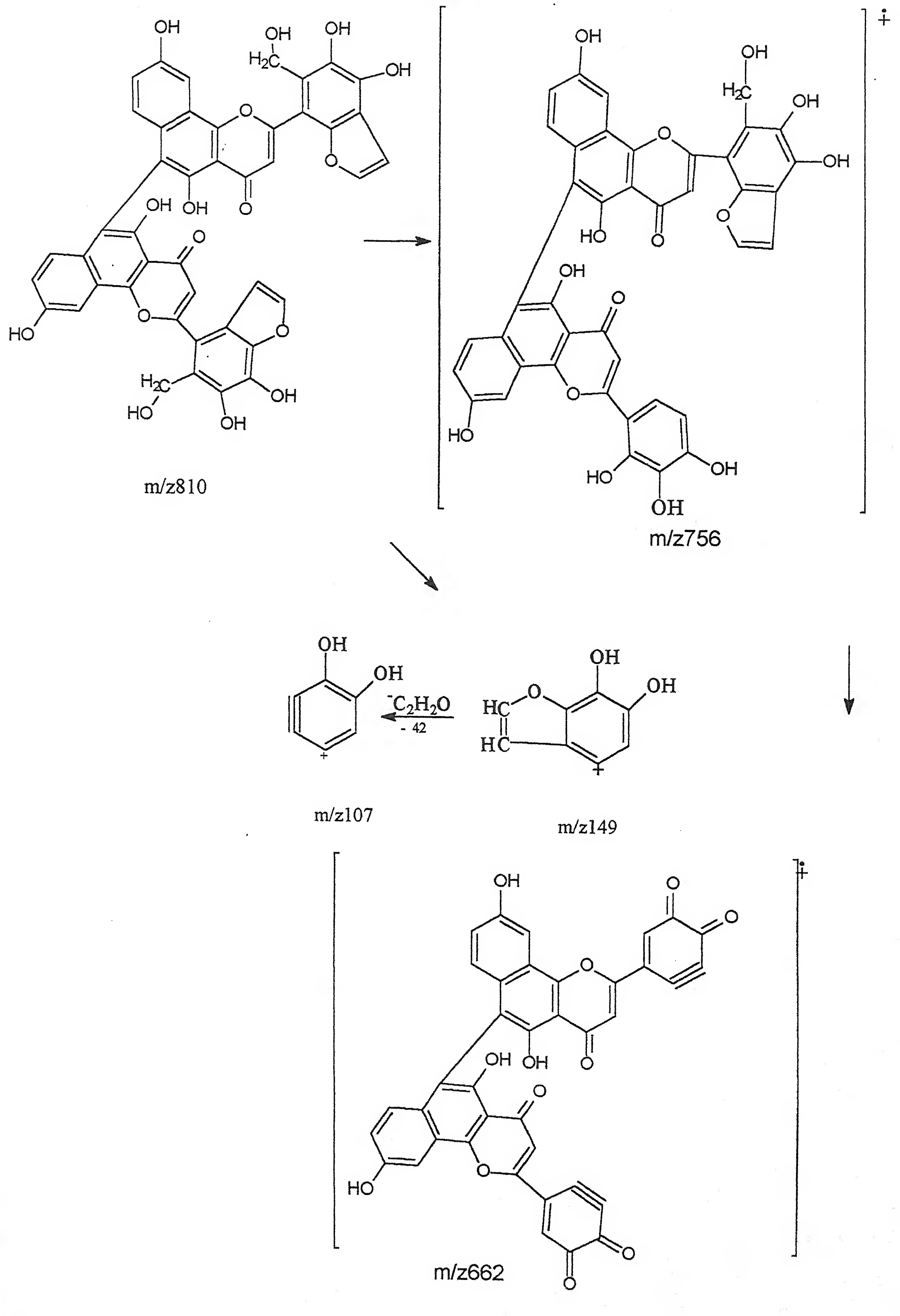


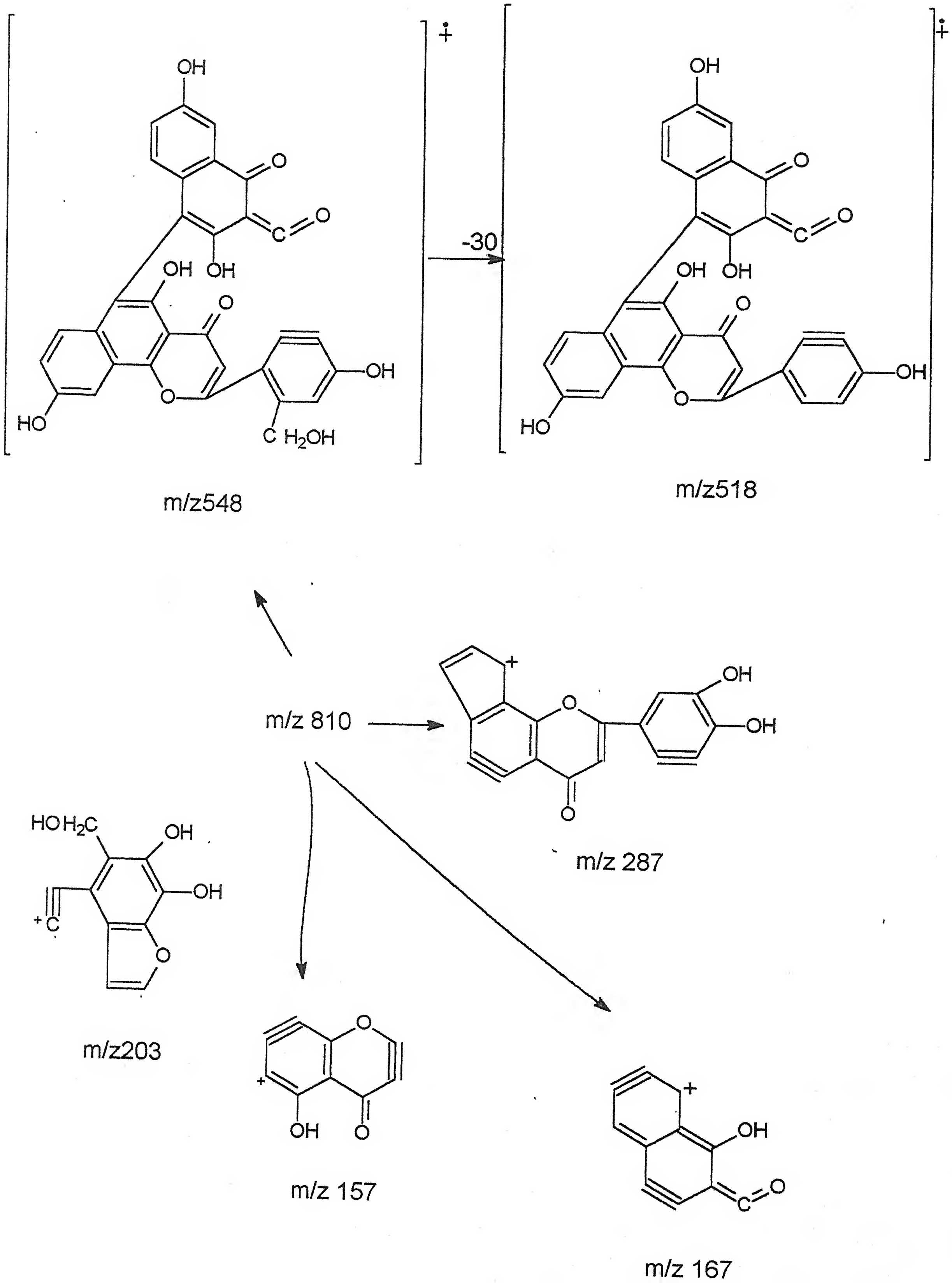
FIG - 9

SCHEME - 3



MASS SPECTRAL FRAGMENTATION OF AP-3

SCHEME – 3 Contd.



EXPERIMANTAL

All melting points were determined on Kofler Bock monoscope and are uncorrected. The UV spectra were recorded on Unichem UV-2 spectrometer. The IR spectra were carried out on Perkin -Elmer Infra - Cord model 157. The ^{1}H and ^{13}C NMR were determined on Brucker 300 specrometer at 300- MHz and 75 MHz respectively in DMSO- d_6 using TMS as internal standard, HMQC spectrum was recorded using standard pulse sequence. The protonated FAB Mass spectrum obtained on 700 Jeol mass spectrometer using Argon/Xenon (6KV, 10MA) as FAB Gas.

Silica gel G (E. merck) and Sephadex LH-20 (Sigma) were used as adsorbent material for thin layer chromatography and column chromatography PLC was carried out on (20 x 20 cm) coated with 24 gm of Silica gel G or cellulose.

The spots on TLC and PLC plates were visualized by exposure on UV light and ammonia vapour as per need. For paper chromatography Whatman No.1 mm chromatography paper were used and spots were detected by UV light/exposure to ammonia vapour.

ISOLATION OF BIFLAVANOIDS

The leaves of *Albizia procera* (Fam. Leguminosae)were collected from the Central Research Farm of Indian Grassland and Fodder Research Institute, Jhansi during monsoon season and the specimen has been deposited in IGFRI herbarium. The leaves were dried under shade and ground, powdered leaves (4 kg) of *Albizia procera* were exhaustively extracted with rectified spirit (3 x 30Lt) by cold percolation and solvent was removed under vacuum. The total extract was

concentrated under reduced pressure to a thick syrupy dark tarry colour mass (220gm).

It was successively extracted with n-hexane, benzene, chloroform, ethyl acetate acetone. The remaining extractive (40 gm) was treated with 0.5% aqueous NaOH and separated into alkali soluble and insoluble part. The alkali soluble fraction was acidified with dil HCl and extracted with ethyl acetate, the ethyl acetate soluble part after consentration under vacuum cromatogrphed over silica gel (60 – 120 mesh) column as follows

Length of column	100 cm
Diameter of the column	2.5 cm
Weight of Si gel	250 gm
Weight of crude extract	1.5 gm

The column was eluted with ethyl acetate and methanol in different ratio. Total 75 fractions (volume 150 ml.) were collected and each was monitored by TLC and the details are presented in Table – 6.

TABLE - 6

S.No.	Fraction	Eluant collected 150ml each		Remark
1.	1 – 5	EtOAc : MeOH	(10 : 0)	Greenish sticky mass
2.	6 – 11	EtOAc : MeOH	(9 : 1)	Light green sticky mass
3.	12 – 18	EtOAc : MeOH	(8 : 2)	Light green solution
4.	19 – 24	EtOAc : MeOH	(7 : 3)	Pale colour solution
5.	25 – 33	EtOAc : MeOH	(6 : 4)	Yielded compound AP-1
6.	34 – 39	EtOAc : MeOH	(5 : 5)	Mixture
7.	40 – 47	EtOAc : MeOH	(4 : 6)	Mixture
8.	48 – 58	EtOAc : MeOH	(3 : 7)	Yielded compound AP-2
9.	59 – 63	EtOAc : MeOH	(2 : 8)	Light yellow solution
10.	64 – 69	EtOAc : MeOH	(1 : 9)	-do-
11.	70 – 75	EtOAc : MeOH	(0 : 10)	-do-

COMPOUND AP-1 -:

Fractions 25 – 33 were pooled owing to their similarity on TLC and purified by PLC using Benzene: Pyridin : Formic acid (36 : 9 : 5) to yield light brown amorphus substance AP-1 (40mg) crystallized from methanol m.p. 204 – 5^{0}C. It gave pink colour with Mg/HCl and appeared fluorescent light blue in presence of ammonia. Its FABMS $[M + H]^+$ 767 corresponded molecular formula $C_{44} H_{30} O_{13}$.

IR SPECTRUM OF COMPOUND AP-1 :-

ν(KBr) max

3462	O-H Stretching
1652	> C = O
1360	Gem dimethyl grouping
1282, 1216	C – O- C Vibration
1150	C – O bending vibration
888, 710, 684	*cis* olefinic bonds

HETERONUCLEAR MULTIPLE QUANTUM COHERENCE (HMQC) OF AP-1-

δ_C/δ_H	
103.4/6.6	(C-3/H-3)
99.1/6.21	(C-6/H-6)
94.2/6.49	(C-8/H-8)
113.4/7.37	(C-2'/H-2')
29.2/1.21	(C-7''/H-7'')
29.2/1.41	(C-8''/H-8'')

FABMS OF AP-1	767, 709, 692,662,614
	593,518,460,424,392
	365,307,287,273, 216
	202,166,154,138,120

ISOLATION OF AP – 2 :-

The fraction 48 – 58 obtained from column chromatography were mixed on the basis of TLC and removed solvent yielded yellow amorphous compound AP-2 (80mg) crystallized from ethanol m.p. 270-72^{0}C. It answered Shinoda and Borntrager reaction. Molecular formula $C_{44}H_{24}O_{16}$ due to $[M + H]^+$ at 809.

IR SPECTRA OF AP – 2 -:

ν (KBr)max

3421	O-H grouping
2920	C – H stretching vibration
1658, 1620	> C = O
1280,1210	C – O – C Vibration
1150	C – O bending vibration
888,760 712,694	cis olefinic bonding

1850,1610 1150,366	Anthraquinone skeleton

FABMS OF AP – 2 -:

809,781,765,710,646,548,

676,392,310,286,232

167,157,137 (BP), 107

ACID HYDROLYSIS OF AP-2 – :

Compound AP-2 (8mg) in 10 ml EtOH was taken with 40 ml of 7% H_2SO_4 in100 ml round bottom flask fitted with water condenser and refluxed on a water bath for 8 hr. and cooled . On examination by TLC the hydrolyzate showed two spots and separated by PLC using $CHCl_3$: MeOH (9 : 1) solvent system. Upper band with Rf = 0.89 and Lower band Rf = 0.65 which were represented as AP-2' and AP-2"

STUDY OF AP – 2' :-

Compound AP-2' orange microcrystalline substance m.p. 268-70^{0}C and orenge red colour with 5 % methanolic magnesium acetate.

UV SPECTRUM OF AP – 2' :-

λ^{max} MeOH 242 386 486

STUDY OF AP – 2" :-

Cream colour substances m.p. 258 - 60 ^{0}C. It gave cherry red colour with Mg/HCl.

ISOLATION OF COMPOUND AP-3 :-

Albizia procera 3kg fresh leaves were processed with acetone : H_2O (7 : 3) containing 0.1 % ascorbic acid in Bazaz Blender 2-3 times. The total extract was left overnight and filtered through a buchner funnel and reduced to aqueous phase under vacuum. The resulting aqueous phase successively extracted with diethylether and ethyl acetate and these were discarded. The aqueous extract was diluted to 1 : 1 with methanol (350 ml) and passed through column of Sephadex LH-20 (200 x 3 cm.). The washing from 50% methanol were collected and lypholized with freez drier to a brown amorphous substance. It was purified by PLC using on cellulose glass plates of 20x20 cm, 0.5mm thickness, solvent system BAW 4:1:5, Rf 0.85.

STUDY OF COMPOUND AP – 3 :-

AP-3 was dark yellow colour powder (28 mg) melted at 216 - 18^0C. It responded green colour with $FeCl_3$ and dark pink colour with Mg/HCl. Molecular formula $C_{44}H_{26}O_{16}$.

IR SPECTRA OF AP –3 :-

ν (KBr)max

3419	O-H Stretching
2630	C-H stretching
1656	>C = O
1265	C – O stretching
1569	fused system
1163	of furan ring
837	adjacent 2H in olefinic bond

FABMS of AP-3 :-

811, 756, 662,
646, 518, 469, 365
287, 261, 232, 214
185, 157, 149, 137

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Chapter - III

Isolation and characterisation of flavonoids from the leaves of *Bauhinia purpurea*.

Bauhinia purpurea (Linn.) (Fam Leguminosae) is popularly known as Atmatli in Hindi and grows widely in forest of sub Himalayan tract and western peninsula. [Jha, 1995]. It is a moderate sized evergreen tree with a bushy crown leaves 3-6 inches long somewhat longer than broad cleft about half way down. Bark dark grey or brown pink to pale yellow insight [Troup, 1986]. The terminal panicled racemes of large purple, deep rose to lilac flowers appear amongst the foliage from September to December.

The soft grayish brown wood is light weight and quite often used in fabricator of agricultural implement low and inferior construction work and the bark for tanning of hides. The leaves are used as fodder with moderate palatability

.

The literature survey has revealed, that its species *Bauhinia championii* has been reported to contain 5,6,7,5' tetra-*O*-Me, 3',4'-*O*-CH_2-*O* flavone [Chen *et.al.*, 1984] and only 4-*O*- β arabino pyranosyl-*O*-β-D galacto pyranoside chalkone from *Bauhinia purpurea* [Bhartiya *et.al.*, 1979]. Gupta *et.al.* [1980] reported the presence of 4'-α-L- rhamnopyranosyl – β -D glucopyranoside from stems of *Bauhinia variegata*. *et.al.*, [1986] isolated E-docosyl *p*- coumarate from stems of *Bauhinia manca*. No account of chemical investigation on *Bauhinia purpurea* is reported hitherto in literature.

Dried and powdered leaves of *Bauhinia purpurea* were extracted by 70% aqueous acetone containing 0.1% ascorbic acid and the acetone was removed under reduced pressure. The aqueous phase was extracted successively with ether, chloroform and ethyl acetate. On TLC examination of ethyl acetate soluble fraction on Si gel_{254} showed two spots using solvent system (Benzene : Pyridine : Formic acid, 36 : 9 : 5). It was subjected for column chromatography on Sephadex LH-20 column (30 x 2.5 cm). The gradient elution of column with H_2O and

1. What about other fractions
2. Test for flavonoid

methanol yielded number of fractions collected (20 ml) were monitored on TLC and fractions of similar nature were pooled. The collected fractions obtained with H_2O : MeOH.,1:1 and 2:8 yielded compound BP-1 and compound BP-2 respectively.

STUDY OF COMPOUND BP-1 :-

Compound BP-1 crystallized from aqueous methanol as brown amorphous substance, m.p. 190 - 92^{0}C, answering positive test for flavonoids [Geissman, 1955]. It corresponded molecular formula $C_{44}H_{30}O_{14}$ on the basis of FABMS $[M+H]^+$ 783. ?

UV SPECTRUM OF BP-1 -:

λ(MeOH)max	252	270	352
log ε	6.65	6.67	6.6
λ (+NaOMe)max.	232	260	400
log ε	6.65	6.67	6.73
λ (+$AlCl_3$)max	272	302	320
log ε	6.71	6.37	6.79
λ (+$AlCl_3$/HCl)max	262	275	292
log ε	7.07	7.03	6.89
λ (+NaOAc)max	272	337	412
log ε	6.95	6.37	6.78
λ (+NaOAc/ H_3BO_3)max	260	366	
log ε	7.20	7.14	

units why in different solvents

The compound BP-1 showed typical UV spectrum of flavonoid. Further the compound BP-1 exhibited a bathochromic shift of 14 nm in presence of sodium

What is bathochromic shift

How you find out molar ext coefficien

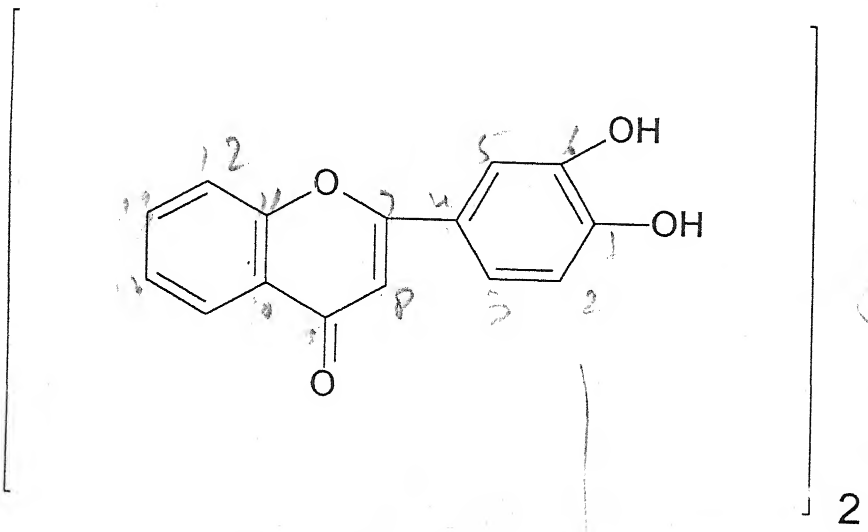
acetate and boric acid revealing the 3'4' *ortho* hydroxylation in B ring of molecule [Mabry *et.al.*, 1970].

BIFLAVONOIDAL NATURE :-

The magnitude of the molar extinction coefficient of BP-1 (6.56) and (6.6) corresponding to λMeOH 252 and 352 were not double to those of typical flavones on consideration this phenomena in conjunction with their molecular formula led to conclude that the BP-1 could be flavone dimer.

PRESENCE OF IMPORTANT FUNCTIONAL GROUP IN BP-1 :-

The structural units inferred with the help of precedental reports [Rao, 1963, Nigam and Saxena, 1981] were as follow 3417cm^{-1} (OH), 2852 cm^{-1} (methoxy), 1656 (Carbonyl) 1508, 1170 cm^{-1} (furan ring) [Finar, 1983] on the basis of UV and IR the tentative structure of BP-1 could be assigned as I.



$C_{14}H_{12}O_6$

What.

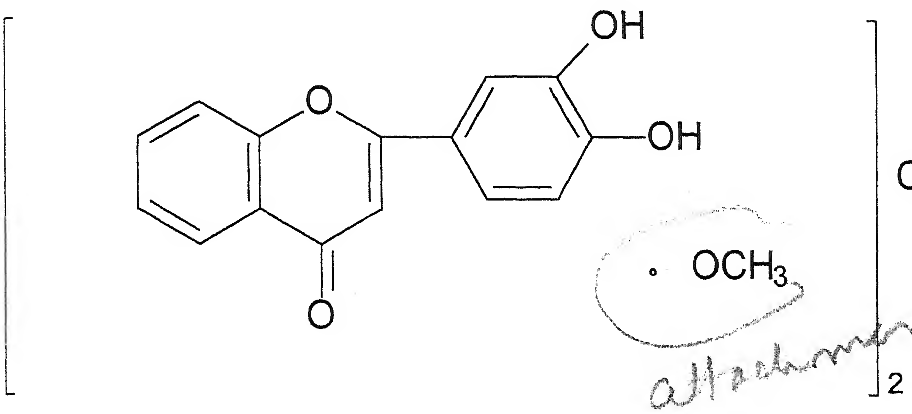
I

How many carbon

PRESENCE OF METHOXY GROUP :-

Appearance of IR peaks at 2852 cm^{-1} and a sharp singlet at δ_H 3.48 integrated for 6 proton led to infer the presence of two methoxy group in BP-1. [Stochmal *et.al.*,2001]

Further ^{13}C NMR of signal at δ_C 61.2 for primary carbon atom supported the presence of methoxy group [Greenham *et.al.*, 2001] (II).

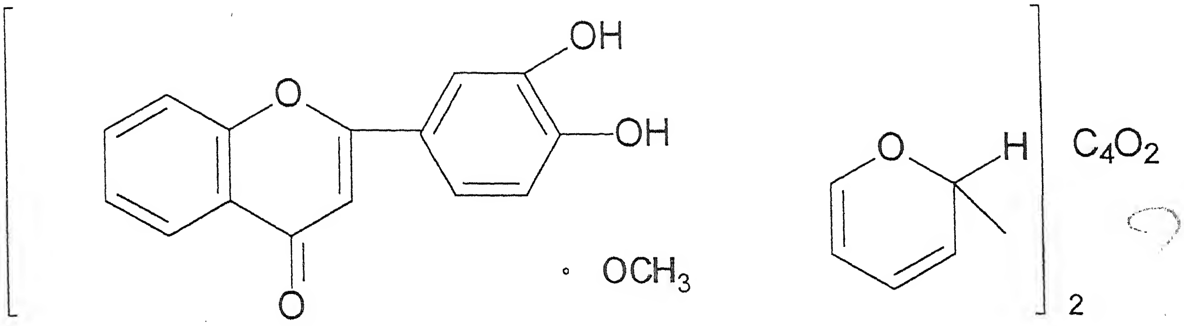


attachment

PRESENCE OF CHROMENE RING -:

IR absorbance at 882, 767 and 694 were indicative of *cis* olefinic double bond of chromene bond, [Lakshmi, *et al.* 1974] besides this the ^1H NMR of BP-1 displayed chemical shifts as doublets at δ_H 7.3 (2H, d, J=8.1 Hz) and δ_H 6.7 (2H,d, J=8.1 Hz) each for two protons. Appearances of a singlet at δ_H 1.21 for 6 proton 2 x CH_3 and a singlet at δ_H 1.77 for two protons adjacent to oxygen function were the diagnoistic feature for the presence of two mono methyl grouping with oxygen function and olefinic protons, of chromene ring [Saxena and Srivastav, 1993]. Presence of chromene ring was further corroborated by the observance of signal in ^{13}C of BP-1 at δ_C 77.8 for quaternary-*O*- bound carbon and high field aliphatic signal at δ_C 27.6 and δ_C 114.4 and δ_H 130.9 for olefinic C atom of chromene [Yankep *et.al.*, 2001]. III.

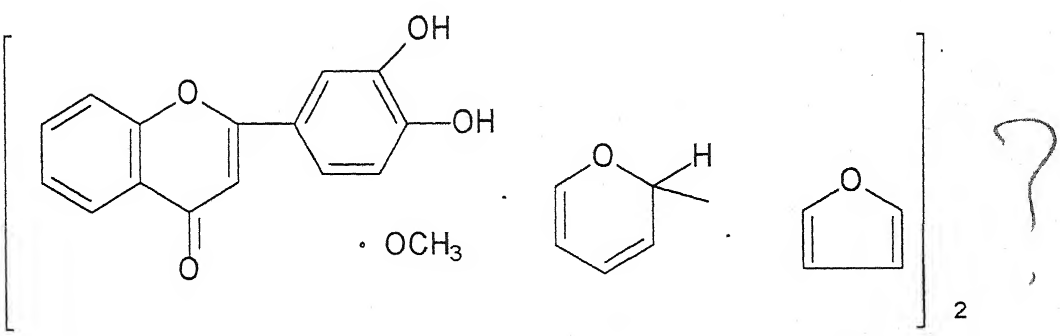
What is chromene ring



III

PRESENCE OF FURAN RING :-

The IR peaks at 1508 ,1170 cm^{-1} were indicative to furan moiety in molecule of BP-1 [Finar, 1983]. The presence of two doublet at δ_H5.99 (2H,d, J=1.2 Hz,) and δ_H 6.25 (2H,d, J=1.2 Hz) in the ^{1}H NMR and two signals at δ_C 130.1 and δ_C 115.2 in ^{13}C NMR of BP-1 corroborated the presence of furan moiety in molecule. [Moriyasu *et.al.*,1998] IV.



IV

STUDY OF ^{1}H NMR OF BP-1 :-

The ^{1}H NMR of BP-1 at 300 MHz (Fig.- 1) displayed the signals for 30 protons which were ascribed on the basis of precedents in literature [Silverstein

et.al., 1974, Jackman *et.al.*, 1969] Table-1. The sharp singlets at δ_H 6.47 and in highly deshielded region at δ_H 7.28 accounting for two protons each for H-3 and H-2' position respectively. A D_2O exchangeable signal at δ_H 8.8 was integrated for four protons which led to concluded the 3',4' hydroxy on both the flavone units. The reason for appearance of other chemical shift have been described in preceeding section.

Table – 1

S.N.	Chemical Shift	Pattern	*J* value Hz.	No. of protons	Assignments
1.	6.47	s	-	2-	H-3
2.	7.29	s	-	2-	H-2'
3	6.7	d	8.1	2-	H-4''
4.	7.3	d	8.1	2-	H-5''
5.	1.77	s	-	2	H-6''
6	1.21	s	-	6	H-7''
7.	5.99	d	1.2	2	H-4'''
8.	6.25	d	1.2	2	H-5'''
9.	3.48	s	-	6	O-CH_3
10.	8.8	s	-	4	OH 3', 4' D_2O exchangeable

STUDY OF ^{13}C NMR OF BP-1 :-

The signals in ^{13}C NMR of BP-1 using DMSO- d_6 at 75 MHz and the structural units were assigned from available literature [Agarwal and Bansal, 1989] have been shown in Table– 2, Fig – 2.

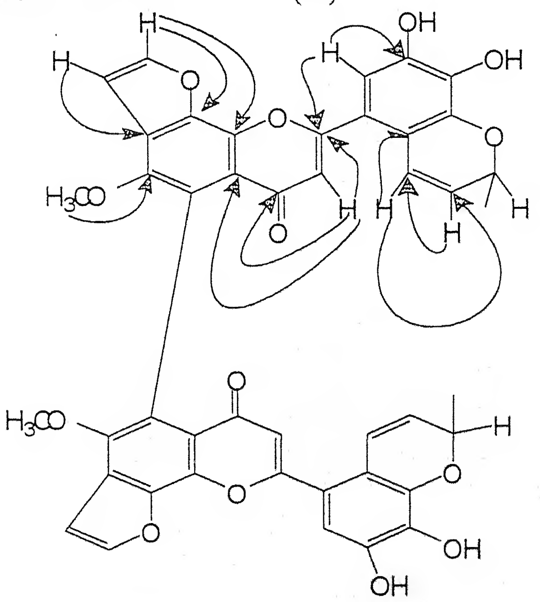
Table – 2

S.No.	Chemical Shift	Assignments
1.	164.0	2
2.	104.0	3
3.	181.0	4
4.	129.2	5
5.	161.1	6
6.	138.2	7
7.	157.3	8
8.	160.1	9
9.	104.8	10
10.	128.4	1'
11.	122.2	2'
12.	145.8	3'
13.	149.7	4'
14.	113.4	5'
15.	155.6	6'
16.	110.4	4''
17.	130.9	5''
18.	77.8	6''
19.	27.6	7''
20.	130.1	5'''
21.	114.4	4'''
22.	61.2	O-CH_3

The ^{13}C NMR displayed 22 Signals for 44 carbons and suggested the symmetric nature of BP-2 [Hamzah *et.al.*, 1997.].The ^{13}C NMR exhibited in addition to signals of flavone moiety the signals of chromene furan and methoxy already described at prerequisite place.

HMBC OF BP-1 :-

In the HMBC spectrum of BP-1 the major correlations observed have been depicted in Fig.-3. The H-3 proton at δ_H 6.47 showed correlation with three quaternary carbon at δ_C 164 (C-2) and δ_C 181 (C-4) with $^2J_{C-H}$ and at δ_C 104.8 (C-10) with $^3J_{C-H}$. Further more two doublets at δ_H 5.99 (H-4''') and δ_H 6.25 (H-5''') for furan at F_2 axis intersected one and two cross peak with δ_C 138.2 (C-6) and δ_C 157.3 (C-8) & δ_C 160.1(C-9) respectively [Moriyasu *et.al.*, 1998]. The characteristic ^1H NMR signal of methoxy at δ_H 3.48 correlated with δ_C 161.1 (C-6). The significant direct connectivity was observed between H-2' proton and δ_C 145.8 (C-3') and indirect connectivity with δ_H 164 (C-2) [Du *et.al.*, 2000]. In the HMBC spectrum long range correlations were observed from H-4'' to C-6' and C-5'' and from the H-5'' to C-4''(V).



V

FABMS OF BP –1 :-

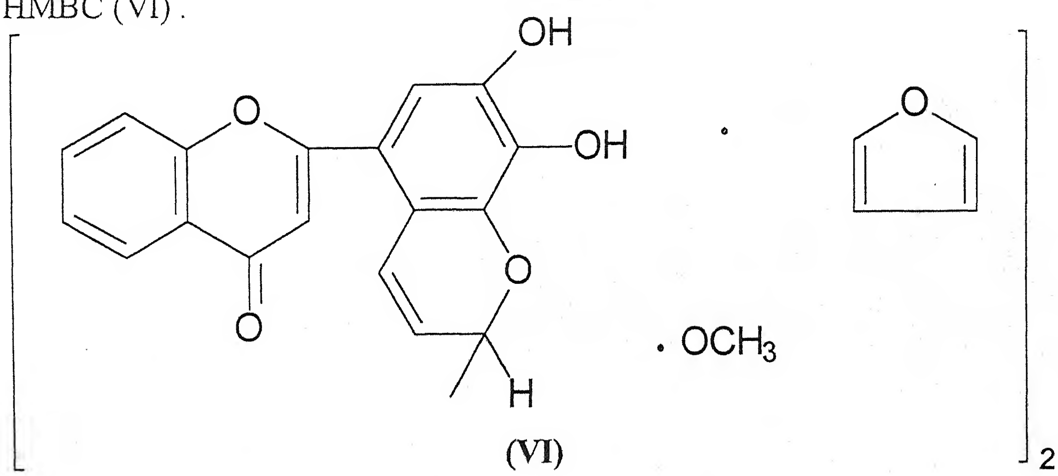
The FABMS (Pos) of BP-1 showed significant molecular ion fragment at m/z 783, 728, 640, 507, 392, 349, 261, 217, 179 the scheme of fragmentation has been shown in a Fig- 4, scheme-1 .

where

The $[M+H]^+$ peak at m/z 783 was corresponded to the molecular formula $C_{44}H_{30}O_{14}$. The presence of an odd electron species at m/z 728 indicated scissoring of chromene moiety. The molecular ion fragment at m/z 640 was suggestive of simultaneous elimination of monomethyl chromene and furan from one of the flavone unit. The RDA with loss of methoxy and furan from A ring yielded molecular ion at m/z 507. Appearance of m/z 392 with a transfer of hydrogen followed by removal of the furan moiety from fragment m/z 349, suggested that BP-1 possessed symmetrical structure. The molecular ion at m/z 217 and m/z 261 were originated from B and A ring of flavone respectively.

POSITION OF CHROMENE RING :-

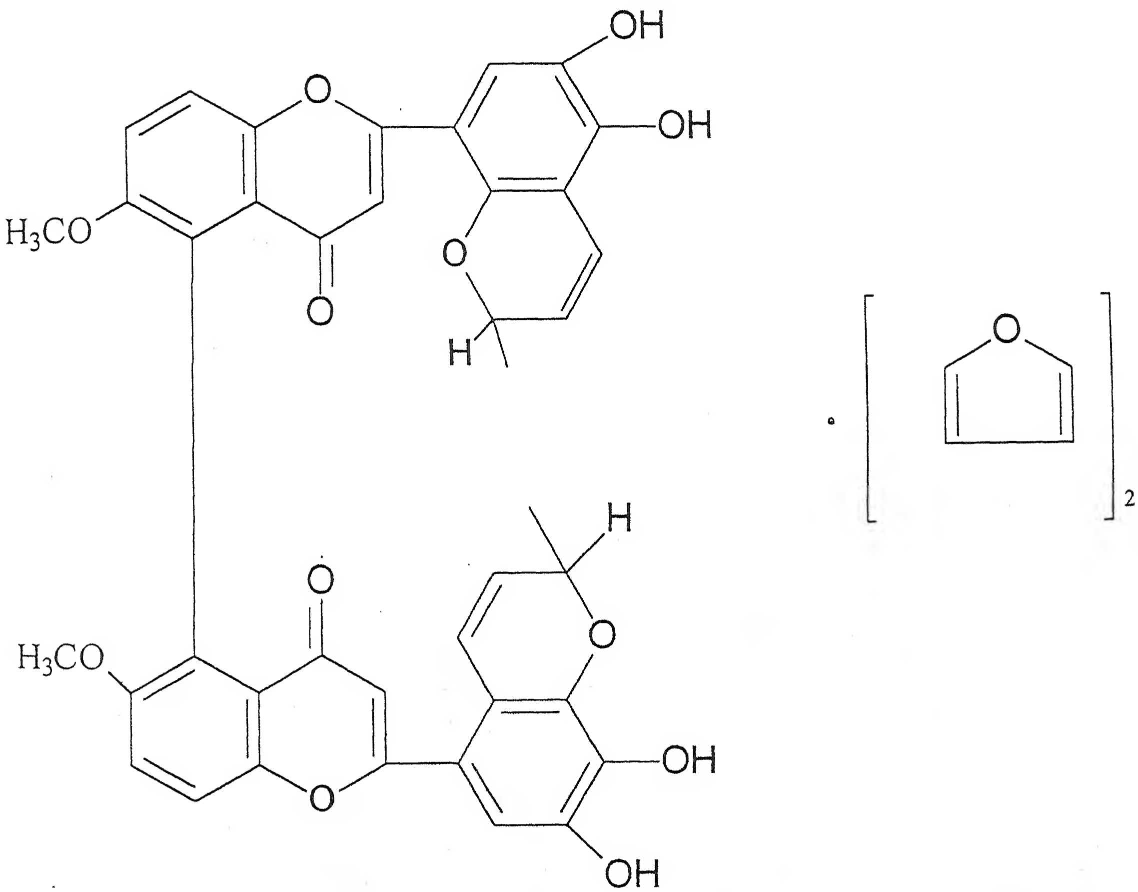
The molecular ion at m/z 217 was indicative of chromene moiety at B ring. The position of two hydroxy group at 3'4' in ring B has already been on page 70. A sharp singlet at δ_H 7.28 for H-2' proton the fusion of chromene at 5',6' position B ring , which was further inferred by $^2J_{C-H}$ by correlation of H-4" proton to C-6' in HMBC (VI) .



(VI)

INTERFLAVONOID LINKAGE :-

The FABMS of BP-1 yielded an species at m/z 507 and m/z 261 was suggestive of interflavonyl linkage through A ring. [Birch *et.al.*,.1967]. The signal observed in ^{13}C NMR at δ_C 129.2 wih downfield shift about 10 ppm was indicative of quaternary carbon atom due to substitution of other flavone unit [Yankep *et.al.*, 2001]. The absence of chelated hydroxy as well as hydrogen bonded proton in ^{1}H NMR spectrum of BP-1 led to deduce the interflavonyl linkage through C-5 [Yankep *et.al.*, 2001] (VII).

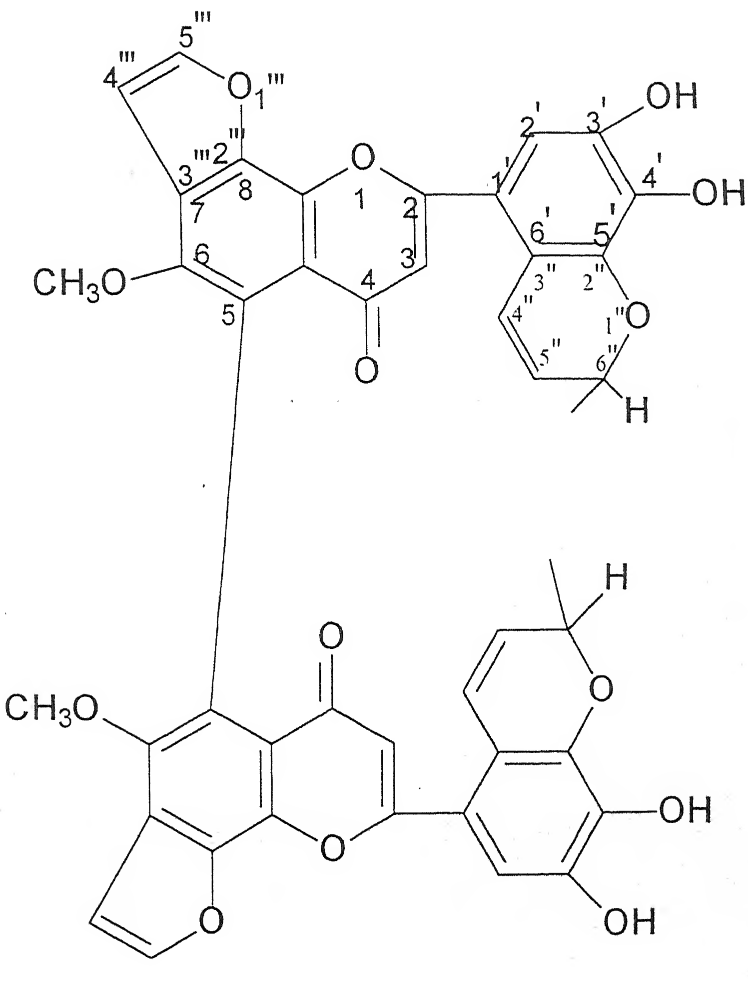


(VII)

POSITION OF FURAN RING :-

The position of chromene ring at 5',6', interflavonyl linkage at C-5 and –O-CH$_3$ at C-6 has already been assigned, leaving the possibility of furan ring at C-7 and C-8 of A ring. It was corroborated further by the presence of two signals at δc 114.4 and δ$_C$ 130.1 [Agarwal and Bansal, 1989] of olefinic carbon and correlation between protons of furan with C-6, C-8, C-10.

All these evidences led to conclude that the compound BP-1 as **I-3',4' , II-3',4' tetrahydroxy I-6, II-6 dimethoxy, I, II difurano (2''', 3''' : 7,8) I,II bis monomethyl chromene (2",3" : 5',6') I-5, II-5 biflavonoid structure** (VIII).



M.F

(VIII) COMPOUND BP - 1

^{1}H NMR SPECTRUM OF COMPOUND - BP - 1

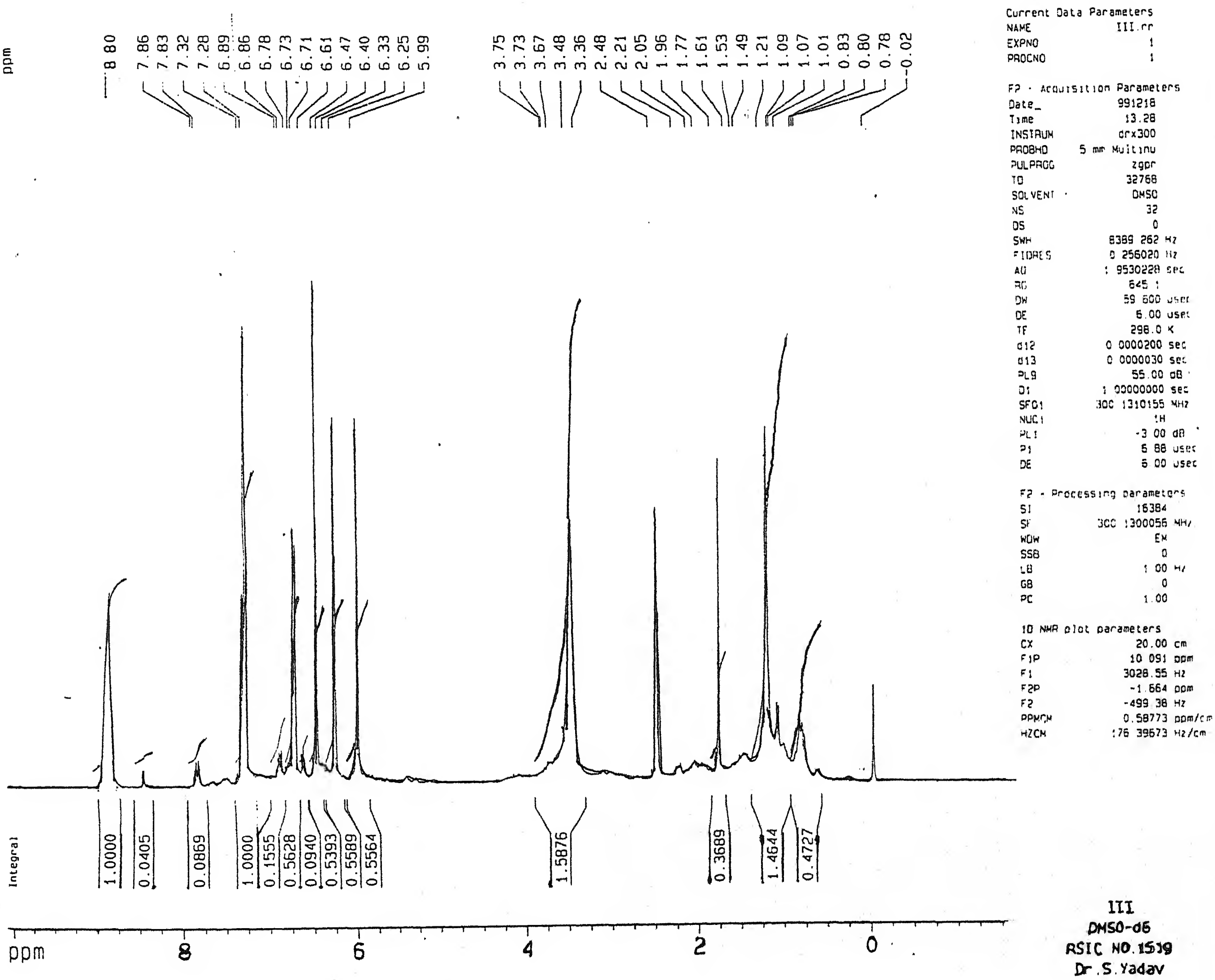


FIG - 1

^{13}C NMR SPECTRUM OF COMPOUND - BP - 1

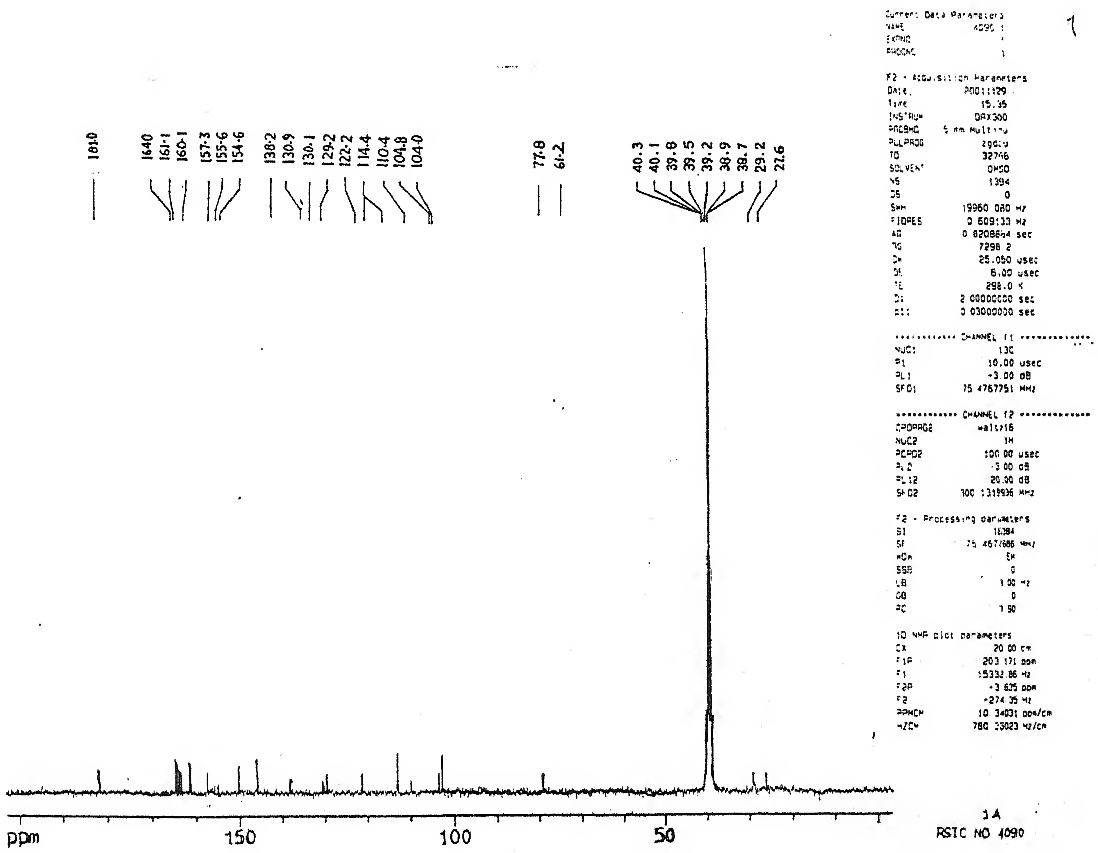


FIG - 2

HMBC SPECTRUM OF COMPOUND BP - 1

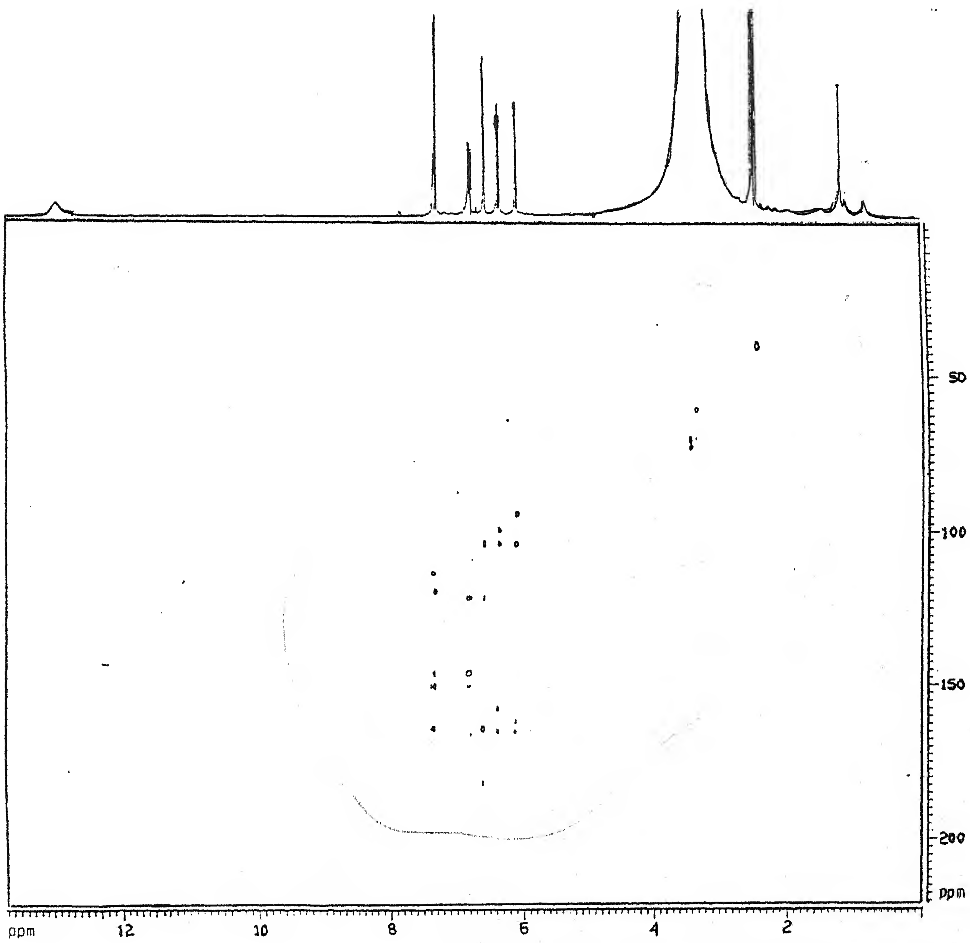


FIG - 3

FAB MASS SPECTRUM OF COMPOUND - BP - 1

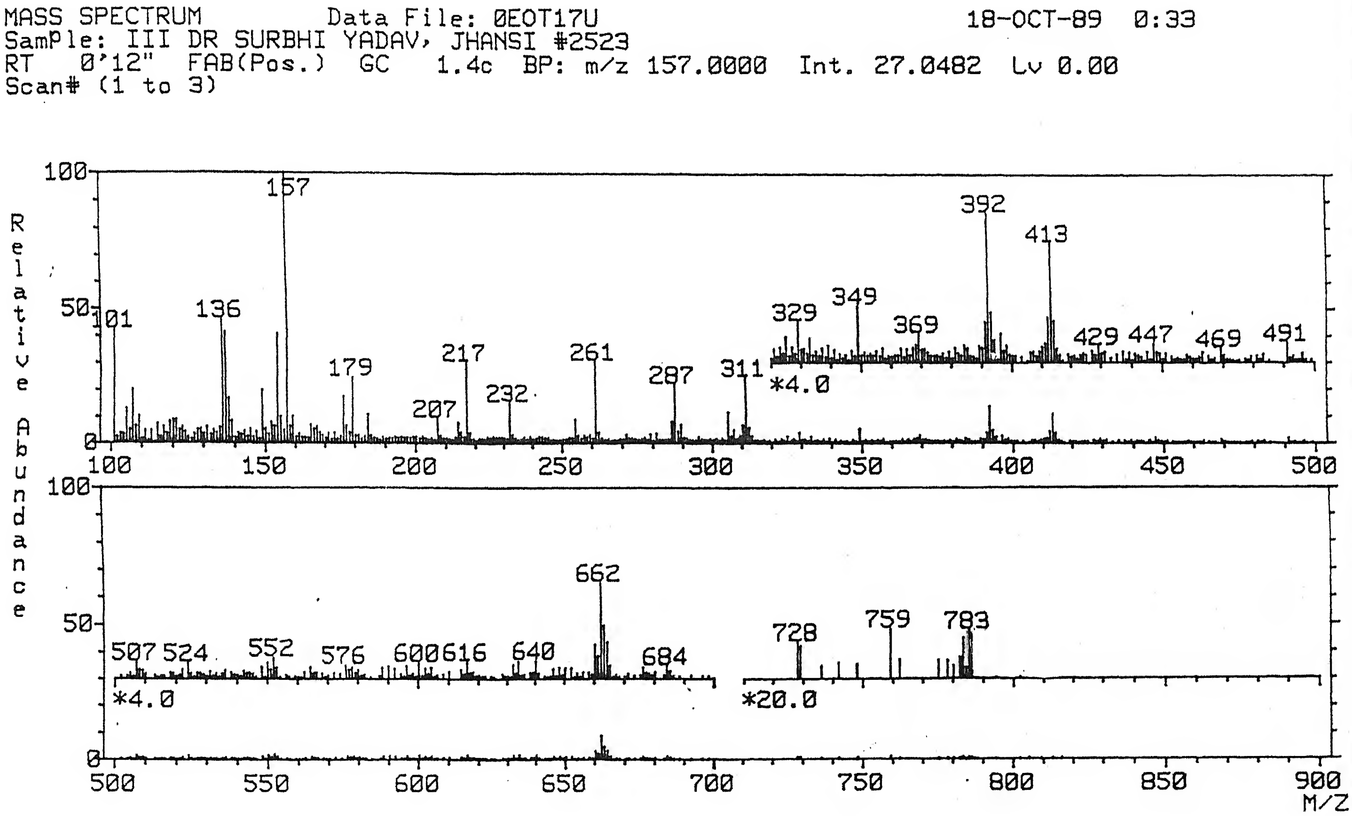
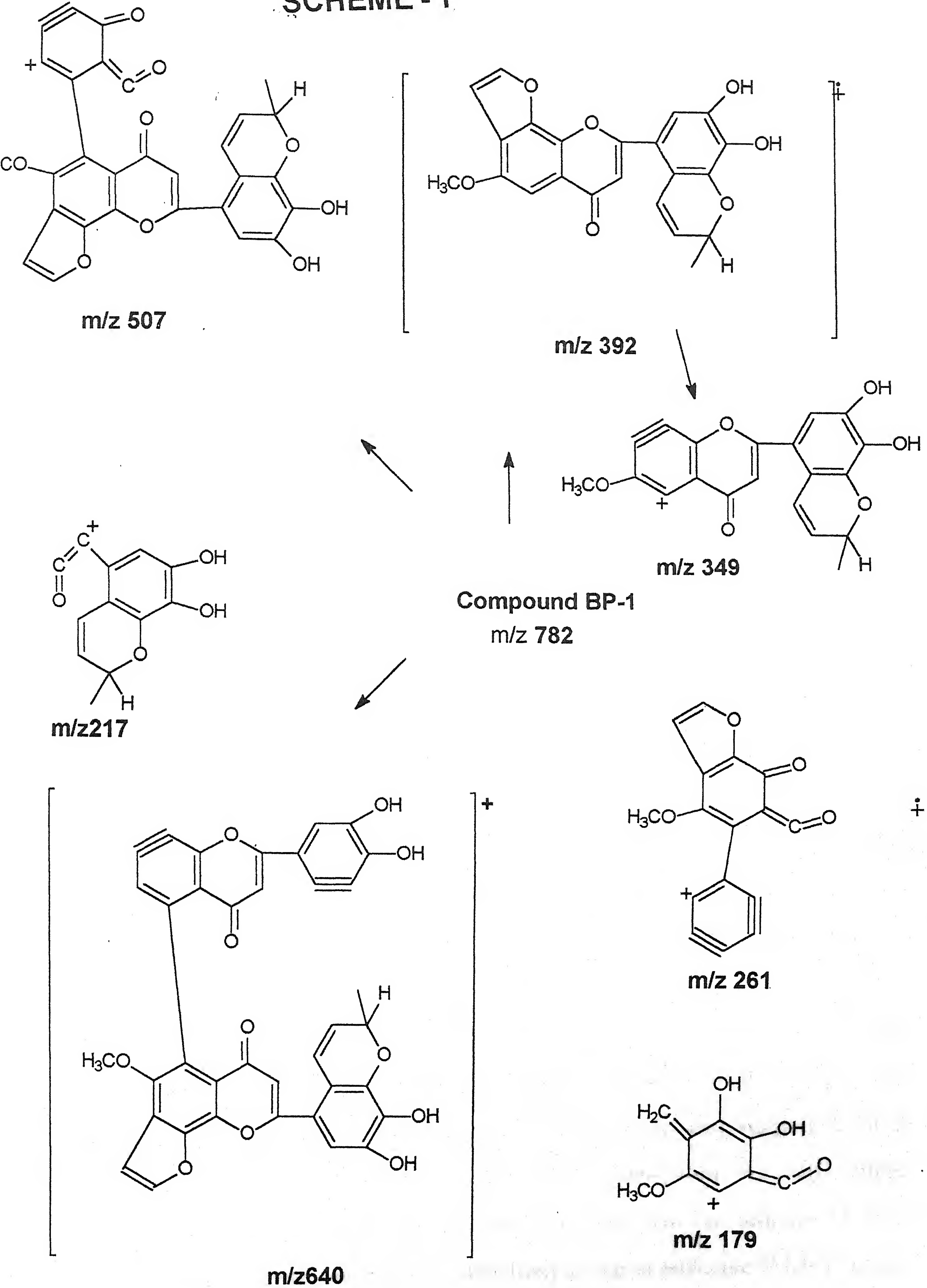


FIG - 4

SCHEME - 1


m/z 507
m/z 392
m/z 349
Compound BP-1
m/z 782
m/z217
m/z 261
m/z 179
m/z640

STUDY OF COMPOUND BP-2 :-

The compound BP-2 dark brown substance crystallised from ethanol, m.p. 250-52^{0} C, showing positive test for flavonoid glycoside [Geissman, 1955, Feigl, 1954].

UV SPECTRUM OF BP-2 :-

The UV spectrum of BP-2 with different shift reagents showed absorption maxima as -

λ (MeOH) max	252	270	352
log ε	6.56	6.4	6.6
λ (+NaOMe)max	232	260	400
log ε	6.65	6.67	6.73
λ (+$AlCl_3$)max	272	302	320
log ε	6.71	6.37	6.79
λ (+$AlCl_3$/HCl)max	272	302	320
log ε	7.07	7.03	6.89
λ (+NaOAc)max	272	334	412
log ε	7.21	7.01	7.19
λ (+NaOAc)max	260	366	
log ε	7.20	7.14	

The UV spectrum of BP-2 with maxima at 270 nm and 352 nm clearly indicate it to be a flavone with C-glycoside [Mitchell, *et.al.*, 2001]. The pronounced shoulder at 320 in the presence of NaOAc was suggestive of a hydroxyl goup at C-4' [Mabry, *et.al.*, 1970]. There were no shift either bathochromically or hypsochromically in $AlCl_3$ spectrum on addition of HCl which ruled out the presence of *ortho* dihydroxy group in molecule [Mabry, *et.al.*,

1970]. The mono hydroxyl substitution at C-4' in the B ring was confirmed by the perusal of ^{1}HNMR of BP-1 later on.

Further the hydroxylation at C-5 was omitted because of the absence of bathochromic shift relative to their band of origin in methanol and no major absorption in presence of $AlCl_3$/HCl.

BIFLAVANOID NATURE OF BP-2 :-

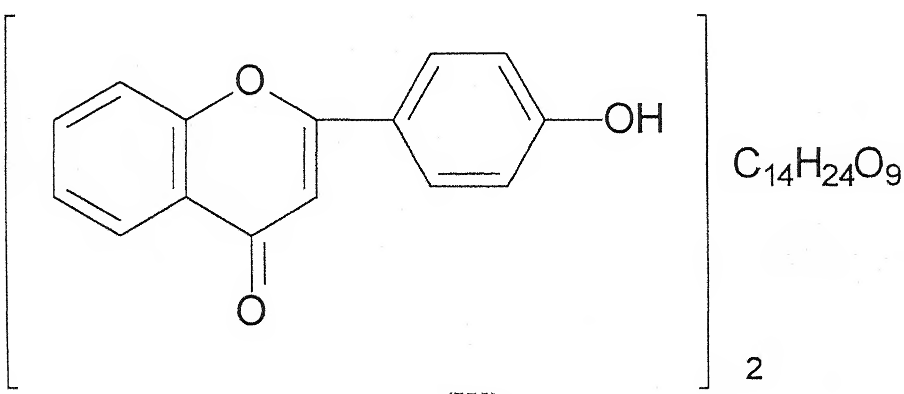
Molar extinction coefficient value 6.56, 6.6 for band at 252 and 352 respectively as calculated from UV spectrum of BP-2 suggested biflavonoid nature [Jackson *et.al.*, 1971]. The high $[M+H]^+$ ion peak in FABMS corroborated the biflavonoid nature of BP-2.

methoxy

PRESENCE OF MAJOR FUNCTIONAL GROUP :-

The important peaks observed were at 3242, 1618, 1568 cm^{-1} in the IR spectrum of BP-2 led to infer the presence of hydroxy group, carbonyl group and aromatic ring respectively on the basis of available literature [Nakanishi, 1962] a broad band at 1183 –1130 cm^{-1} was indication of its glycosidic nature. [Jain, *et.al.*,1990].

On the basis of UV and IR data obtained the basic skeleton of flavone unit of BP-2 could be as IX.



(IX)

The positive Molish's test indicated the presence of sugar unit. The BP-2 was resistant to the acid hydrolysis [Saxena and Shrivastav, 1986] even for longer time showing absence of -*O*- linkage in glycoside and presence of *C*-glycoside.

DEGLYCOSYLATION OF BP-2 :-

The controlled deglycosylation of BP-2 by hydroiodic acid in presence of phenol at 135^{0}C yielded crystalline powder as aglycone designated as BP-2'. It was found homogenous on the solvent system $CHCl_3$: MeOH : H_2O (13 : 7 : 2), Rf = 0.81, m.p.-162^{0}C. It responded characteristic colour reaction of flavone .

IR SPECTRUM OF BP-2' :-

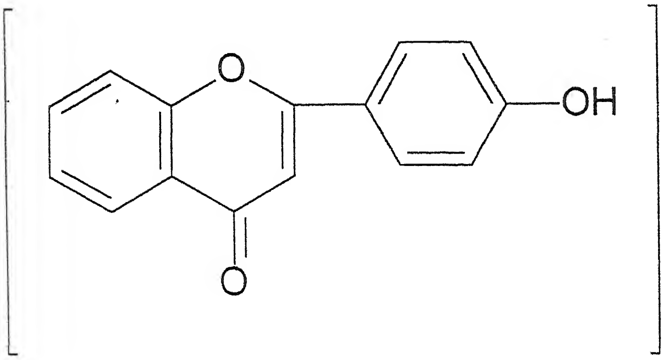
The depicted significant peak at 3265 cm,$^{-1}$ 1665 cm^{-1} and 1640 cm^{-1} in the IR spectrum of BP-2' led to infer the presence of hydroxyl group, carbonyl group and aromatic ring respectively on the basis of available literature [Conley, 1972]

UV SPECTRUM OF BP-2' :-

The UV spectrum of the aglycone exhibited absorption maxima with different reagents as follows.

$\lambda(MeOH)_{max}$	255 sh	311	325
$\lambda(+NaOMe)_{max}$	251 sh	294	380
$\lambda\ (+AlCl_3)_{max}$	248 sh	310sh	327
$\lambda\ (+AlCl_3/HCl)_{max}$	253 sh	310sh	327
$\lambda\ (+NaOAc)_{max}$	257	309	380
$\lambda\ (+NaOAc/H_3BO_3)_{max}$	256	312	328

The UV bonds of BP-2' were indicative of absence of OH at C-3 position. There were not much difference in UV spectrum of aglycone and glycoside. Therefore on the basis of UV spectrum the tentative structure of aglycone could be assigned as (X).



(X)

^{1}H NMR OF BP – 2' :-

The significant signals obtained in ^{1}H NMR of the compound BP-2' and structural units inferred with the help of available literature [Bovey, 1969, Bible. 1965] in Table – 3.

The resonance appearing at δ_H 7.85 [4H, d, J = 8.4 Hz] and δ_H 6.72 [4H, d, J = 8.4 Hz] were suggestive of *ortho* coupled proton at 2'3'and 5'6' respectively of AA' and BB' system. Two *meta* coupled proton in the aglycon for H-8 and H-6 splitted as doublet at δ_H 5.8 [2H, d, J = 3 Hz] and δ_H 6.6 [2H, d, J = 3 Hz] The chemical shift as sharp singlet at δ_H 1.5 integrating for 6 protons indicated presence of CH_3. The appearance of sharp singlet at δ_H 6.64 accounting for two proton led to infer of H-3 proton.

TABLE – 3

S.No.	Chemical Shift	Pattern	*J* value Hz	No. of protons	Assignment
1.	δ 6.64	s	-	2	H-3
2.	δ 5.8	d	3	2	H-6
3.	δ 6.6	d	3	2	H-8
4.	δ 6.72	d	8.4	2	H-2'
5.	δ 7.85	d	8.4	2	H-3'
6.	δ 7.85	d	8.4	2	H-5'
7.	δ 6.72	d	8.4	2	H – 6'
8.	δ 1.5	s	-	6	CH_3
9.	8.85	s	-	2	OH-4'

IDENTIFICATION OF SUGAR MOIETY BY $FeCl_3$ OXIDATION :-

The $FeCl_3$ oxidation of BP-2 after removal of Fe^{+3} and Cl^- by using resins IRC- 120 (H) and IRA – 400 yielded sugar part in a neutral solution [Bhatia *et.al.*, 1966] It was examined by paper chromatography using solvent system BAW (4:1:5) and aniline hydrogen phthalate as detecting reagent. The sugars were identified as glucose and rhamnose in equimolar ratio (1 : 1).

STUDY OF ^{1}H NMR OF BP- 2 :-

The observed chemical shifts in ^{1}H NMR of BP-2 in DMSO – d_6 at 300 MHz (Fig- 5) and structural unit inferred with the help of literature have been recorded in Table - 4.The presence of two sugar moieties in ^{1}H NMR were clearly

evidenced by chemical shifts as multiple at δ_H 3.2–3.8 integrating for 10 protons. The sharp singlet in upfield region appearing at δ_H 0.83 for 3 proton of methyl group could be ascribed for CH_3 of rhamnose [Gohar *et.al.*, 2000].

The presence of rhamnose could also be complemented by Paper Chromatography results after ferric chloride oxidation of BP-2. Therefore another sugar in molecule obviously was assignable as glucose. The two equitorial anomeric protons of both the sugar moieties were indicated by the presence of doublet at δ_H 4.67 having low coupling constant (J = 1.5Hz) [Carnat *et.al.*,1998] between 1'' and 2'' and 1''' and 2'''.

The two *ortho* coupled protons resonating as AA' and BB' system at δ_H 7.9 (4H, d, J = 8.4 Hz) and δ_H 6.8 (4H, d, J = 8.4 Hz) were displayed by doublets for 2'6' and 3'5' respectively .

TABLE – 4

S.n.	Value	Pattern	J value Hz	No. of proton	Assignment
1.	δ_H 5.9	d	1.7	2	H-6
2.	δ_H 6.5	d	1.7	2	H-8
3.	δ_H 6.8	d	8.4	2	H-2'
4.	δ 7.9	d	8.4	2	H-3'
5.	δ 7.9	d	8.4	2	H-5'
6.	δ 7.9	d	8.4	2	H-6'
7.	1.53	d	-	2	CH_3
8.	0.83	d	-	2	1'''rhamnose
9.	4.67	d	1.5	2	anomeric protons of sugars
10.	8.75	s	-	2	OH-4'

The high field signals splitting as doublets at δ_H 6.5 (2H, d, J = 1.7 Hz) and δ_H 5.9 (2H, d, J = 1.7 Hz) were exhibited because of protons at 6 and 8 position at ring A and shift appearing as a sharp singlet at δ_H 1.53 equivalent for 6 protons corroborated the presence of 2 x CH_3 group on A ring of I & II units of biflavonoid.

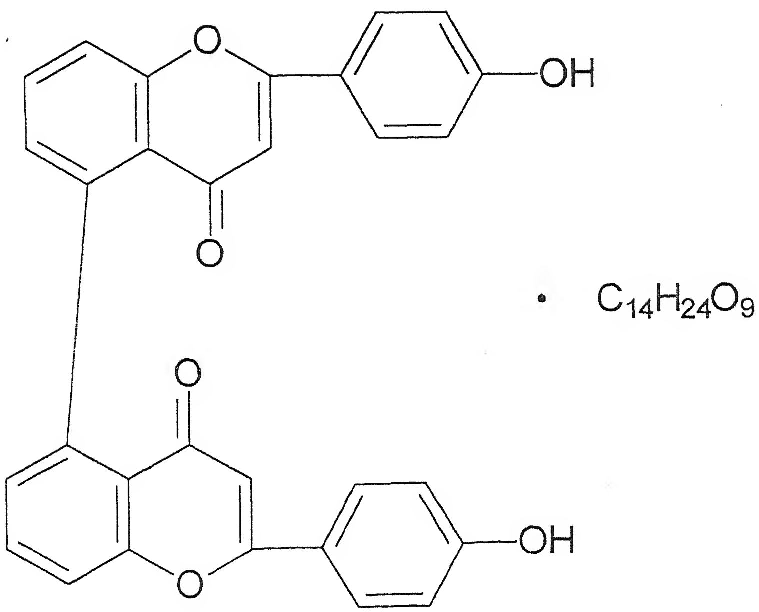
FABMS OF BP-2 :-

The important species assignment to fragments have been shown in scheme - 2 Fig -6. The $[M + H]^+$ 811 corresponded the molecular formula $C_{44}O_{15}H_{42}$.

The odd electron species at m/z 662 was produced by abstraction of rhamnosyl and hydrogen from C-6,' followed by elimination of CH_3 group yielded into molecular fragment at m/z 648, breaking of inter flavonyl linkage resulted into formation of molecular ion at m/z 413. The Retro Diel's Alder cleavage with loss of methyl group produced fragment at m/z 515. The appearance of molecular ion at m/z 261 and m/z 289 enabled to assign attachment of both monomeric unit through A ring. The molecular ion peaks at m/z 136 and 157 were fragmented from A ring where as B ring of flavonoid yielded molecular ion m/z 107.

INTER FLAVONOID LINKAGE :-

The species obtained by FABMS at m/z 515 , 289 and 261 provided an option that both the flavone units were interconnected through A ring. It was well supplemented by the absence of chelated proton in low field suggested the interflavonoid linkage through C – 5.



(XI)

ATTACHMENT OF THE SUGAR MOIETIES :-

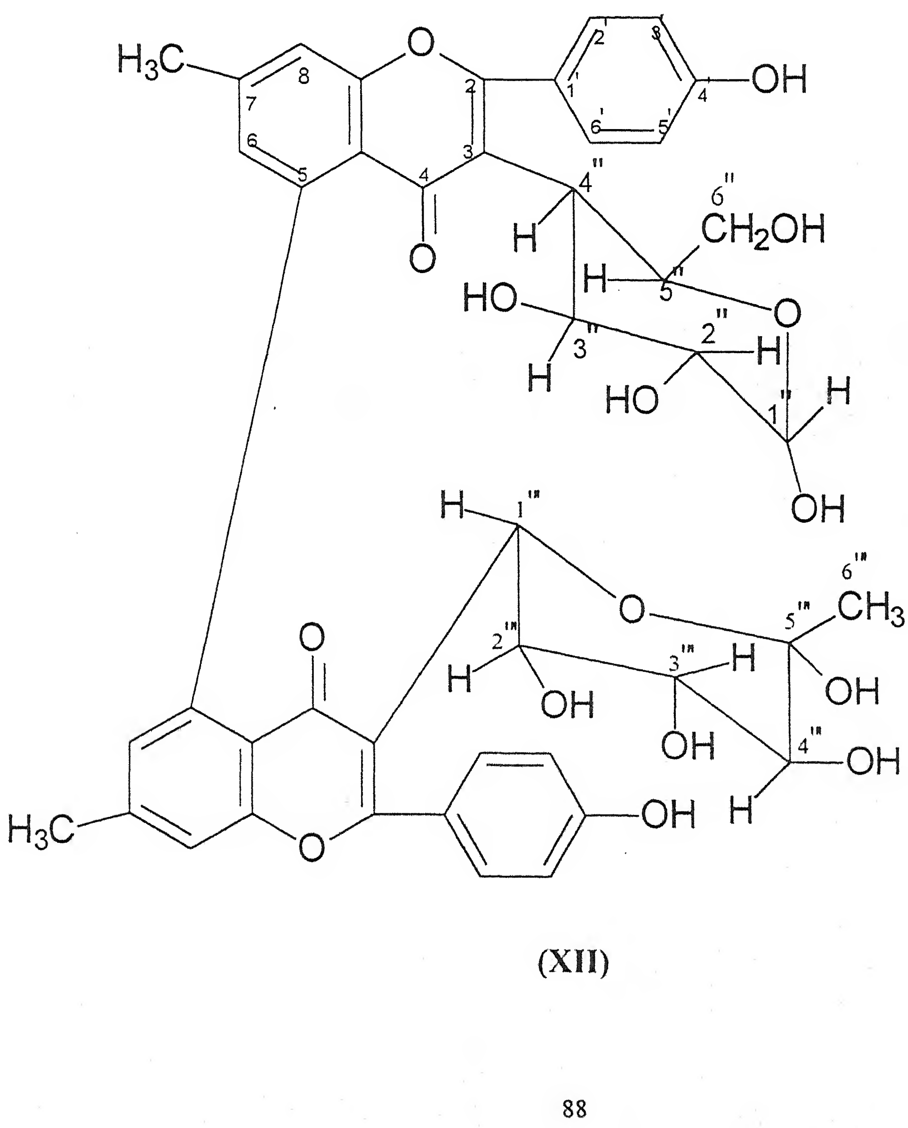
There were two possibilities of attachment of the sugars to the aglycone (i) both the sugars were attached separately on C atom of aglycone (ii) both glucose and rhamnose as disacchrides were attached to the one C atom.

However the perusal of NMR spectra of BP-2 clearly indicated that the chemical shift, experienced by both flavone unit were exactly same. Absence of C-3 proton signal in BP-2 and appearance of sharp singlet at δ_H 6.64 in aglycone BP-2' integrating for two protons gave an option the glucose and rhamnose were attached seperately at C-3 position of two different flavones. The yield of FABMS fragment at m/z 515 after Retro Diel's Alder cleavage that the rhamose was attached at C-3 position of one of the flavone, molecular ion at m/z 413 complemented that both sugars were attached on C-3 of each flavone.

POSITION OF METHYL GROUP :-

The fragments at m/z 289, m/z 157 suggested the presence of CH_3 group at A ring of both flavonoid unit. The presence of *meta* coupled protons of A ring have already indicated the presence of CH_3 at C-7 because at C-5 position involved in interflavonyl linkage.

On the basis of aforesaid deliberation the structure of BP-2 could be assigned as **I - 4', II - 4' dihydroxy I –7, II – 7 dimethyl I – 3, α –L rhamnoside II – 3, α –D glucoside I-5, II-5 biflavonoid** (XII)



(XII)

^{1}H NMR SPECTRUM OF COMPOUND - BP - 2

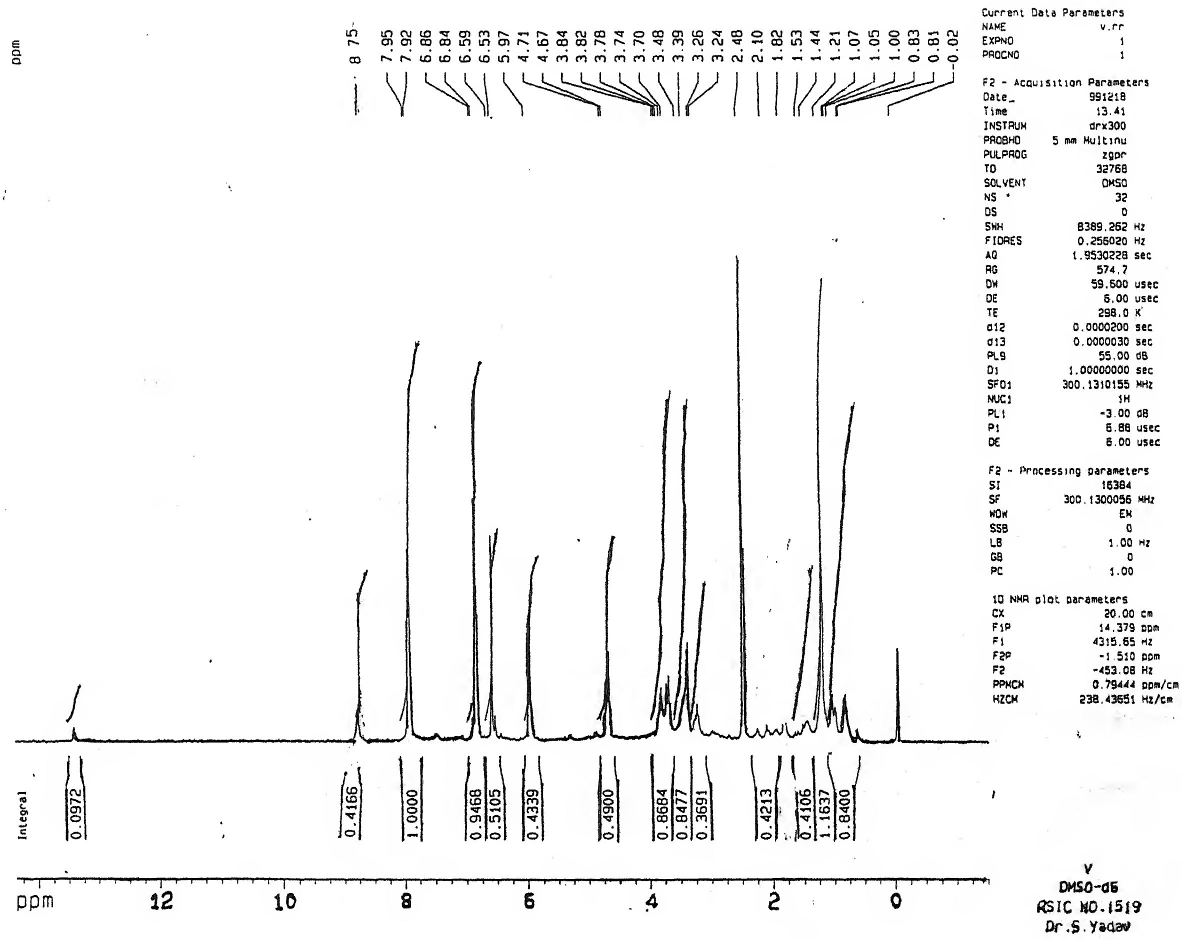


FIG - 5

FAB MASS SPECTRUM OF COMPOUND - BP - 2

MASS SPECTRUM   Data File: 0EOT17V   19-OCT- 0 10:41
Sample: IV DR SURBHI YADAV, JHANSI #2523
RT   0'12"   FAB(Pos.)   GC   1.4c   BP: m/z 136.0000   Int. 47.6938   Lv 0.00
Scan# (1 to 3)

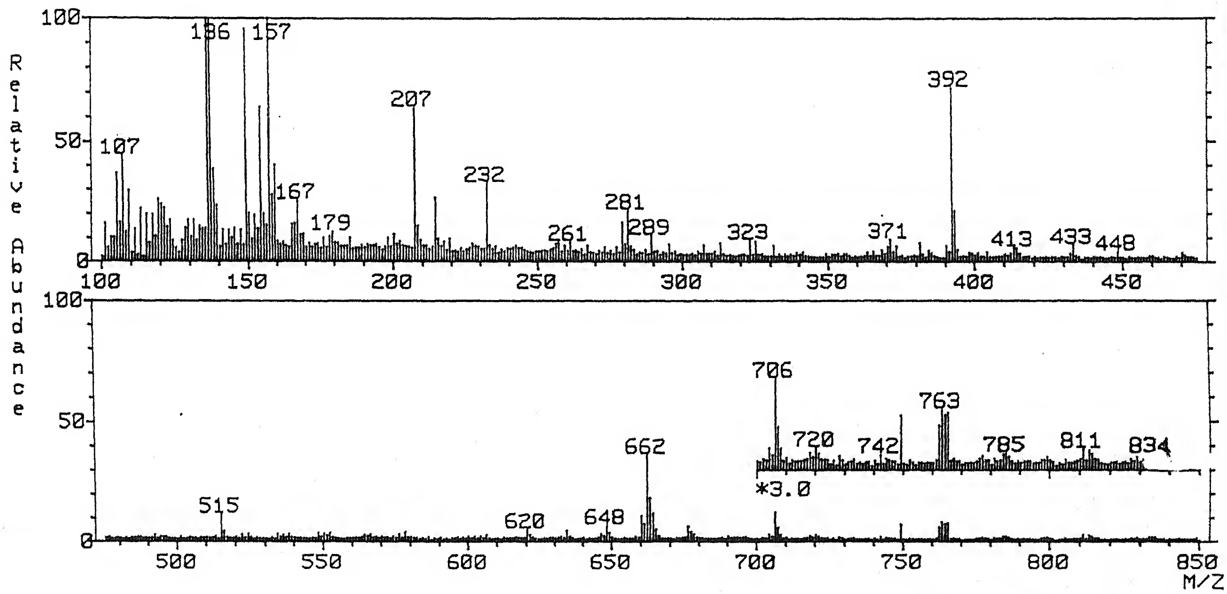
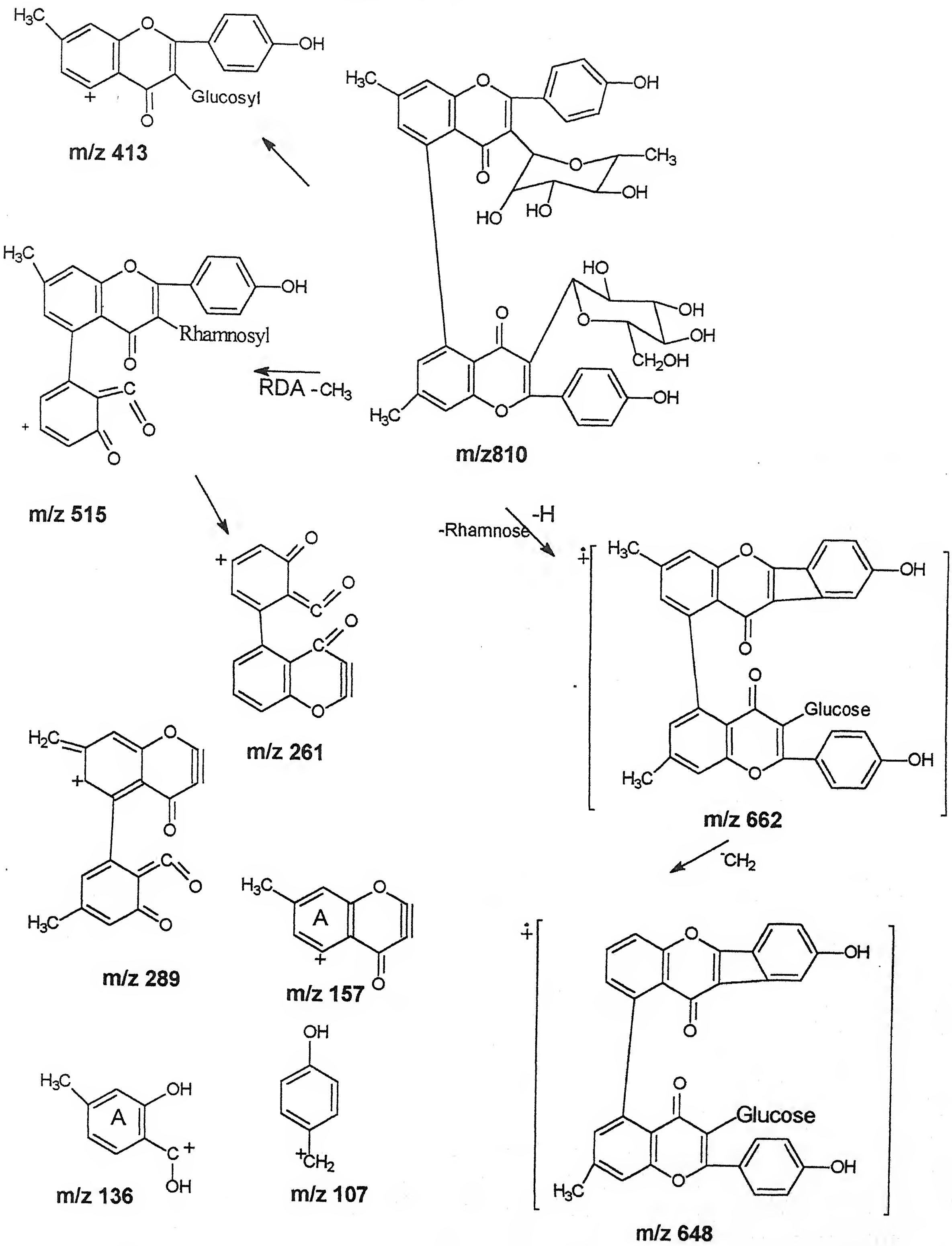


FIG - 6

SCHEME - 2



MASS SPECTRAL FRAGMENTATION OF BP-2

before chromo

EXPERIMENTAL

The leaves of *Bauhinia purpurea* (Fam-Leguminosae) were collected from the Central Research Farm of Indian Grassland and Fodder Research Institute, Jhansi during monsoon season and the specimen has been deposited in IGFRI herbarium.

The air dried powdered leaves (2Kg) were extracted with 70% acetone (3 x 1 Lt.) containing ascorbic acid by cold percolation and solvent was removed under reduced pressure.

The aqueous phase was washed subsequently with ether, chloroform and ethyl acetate. The ethyl acetate soluble fraction showed two florescence spot on Silica gel GF $_{254}$ (solvent system, Benzene : Pyridine : Formic acid , 36 : 9 : 5). It was purified on Sephadex LH-20 column (30 x 2.5 cm) pre equilibrated with water.

The column was eluted with water and methanol in different ratio. Total 82 fractions were collected and each were monitored by TLC.

Table – 5

S.No.	Fractions	Eluant collected 150 ml.	Remark
1.	1-4	Pure Water	Colourless
2.	5-9	H_2O : MeOH (9 : 1)	Greenish solution
3.	10-16	H_2O : MeOH (8 : 2)	Yellow
4.	17-22	H_2O : MeOH (7 : 3)	Yellow
5.	23-29	H_2O : MeOH (6 : 4)	Yellow
6.	30-35	H_2O : MeOH (5 : 5)	Isolatd BP-1
7.	36-42	H_2O : MeOH (4 : 6)	Yellow
8.	43-50	H_2O : MeOH (3 : 7)	Isolated BP - 2
9.	50-62	H_2O : MeOH (2 : 8)	Light Yellow Solution
10.	63-71	H_2O : MeOH (1 : 9)	Light yellow solution
11.	72-82	Pure : MeOH	Light yellow solution

COMPOUND BP – 1 :-

Fractions 30 – 35 were pooled due to their similarity on TLC and purified by PLC using BPF (36 : 9 : 5) to yield substance BP-1 (82 mg) crystallized from aqueous methanol m.p. 190 - 2^0C .It gave pink colour with Mg/HCl : Molecular formula $C_{44}H_{30}O_{13}$.

INFRA RED SPECTRUM OF BP-1 - :

ν (KBr)max

3417	O-H stretching
2852	$O\text{-}CH_3$
1656	> C = O
1508, 1170	fused furan ring
882, 694	Two adjacent Hydrogen atom in Benzene ring.

HMBC OF BP – 1 :- Major Correlation of BP - 1

δ_H	Correlates with δ_C
δ_H 6.47 (H-3)	δ_C 104.8 (C-10), δ_C 164 (C-2)
	δ_C 180 (C - 4)
δ_H 5.99 (H-4''')	δ_C 138.2 (C-7)
δ_H 6.25 (H-5''')	δ_C 157.3 (C-8), δ_C 163.9 (C-9)
δ_H 3.48 ($O\text{-}CH_3$)	δ_C 163 (C-6)
δ_H 7.29 (H-2')	δ_C 145.8 (C-3'), δ_C 164 (C-2)
δ_H 6.7 (H-4'')	δ_C 155.6 (C-6'), 130.9 (C-5'')
δ 7.3 (H-5'')	δ_C 110.4 (C-4'')

Is it not repetition

FABMS OF BP – 1 :-

783,759,728,662,640,600,552,524,507,413,

392, 349,329,311,287 261, 217,207,179,157,136

COMPOUND BP – 2 :-

Fractions 50 – 62 were cooled and lipholised as dark brown substance (105 mg.) m.p. 205 -7^{0}C. Molecular formula C_{44} H_{42} O_{15}. It gave positive Molish test, and pink colour with Mg/HCl.

DEGLYOSYLATION OF BP – 2 :-

10 mg of BP –2 was taken in 50 ml flask, 0.5 ml HI in phenol was added gradually with cooling. The mixture was gently refluxed (135-137^0) for 8 hr. and was poured into $NaHSO_3$ solution in water. Crystallization of the product from MeOH gave yellow crystal of aglycone designated as BP-2'.

R

STUDY OF BP – 2' :-

The yellow crystalline compound, m.p. 291-92^{0}C. It gave orange pink colour with Mg/HCl.

$FeCl_3$ OXIDATION FOR IDENTIFICATION OF SUGARS :-

10 mg of compound BP-2 and 0.1g $FeCl_3$ in 0.8 ml of water yielding a dark colour complex which was heated at 115^{0}C for 15 minutes and then 125^0 for 6 hr. The mixture was diluted with water and the dark colour complex was filtered. The yellow colour filterate was treated successively with resins IRC- 120 (H) and IRA-400 to remove Fe^{+++} and Cl^- ion and the neutral solution was concentrated in vacuum and examined by paper chromatography with the authentic specimen of

R

sugars as reference on Whatmann no. 1 filterpaper in butanol : acetic acid : water (4 :1 :5 v/v) solvent system. The paper chromatogram was air dried, sprayed with aniline hydrogen phthalate and kept in hot air oven at 120^{0}C for half an hour. The chromatogram showed two spots corresponding to the glucose (Rf 0.18) and rhamnose (Rf 0.37) was obtained.

IR SPECTRUM OF BP-2 :-

ν (KBr)max	3417	OH group
	2920	- CH stretching
	1659	> C = O
	1618, 1568	Aromatic ring.
	1183, 1130	Glycosidic nature

IR

FABMS OF BP – 2 :-

811 , 785 , 763 , 742, 720 , 706, 662, 620, 515 ,433, 392 , 323, 289, 261 , 232 , 207, 179, 167, 157, 136

R

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Chapter - IV

Isolation and characterisation of flavonoids from the leaves of *Leucaena diversifolia*

Leucaena diversifolia (Schltdl) is one of the most widely cultivated species of genus Leucaena after *Leucaena leucocephela*. It is a very fast growing. [Benth, 1842]. Its young branches are smooth with rougher, grey brown trunk, with brown vertical feissure. Leaves are pinnate 16-24 pairs, 5-8 cm long, densely covered with minute white hair, leaflets 4-5 mm. long, 0.8 - 1mm wide, linear oblong acute at apex, strongly asymmetric at base glabrous except hairy margins. Petiole glands are single flat sessile, discoid or shallow cup shaped, elliptic nectary. Flower heads are 11-15 mm in diameter. The buds are loosely packed 45-90 flower per heads.

Leucaena diversifolia has 10-13cm long, 13-16mm wide narrowly linear oblong flat 1-6 pods per flower head. It has been used for both fodder and wood. Its light crown makes it an ideal species for shade over perennial crops. [Hughes, 1998].

The thorough screening of literature has revealed that *Leucaena diversifolia* had lower palatability, digestibility and high phenolics level indicating poor fodder quality [Bray, 1987 . Austin *et. al.*, 1991].

Fresh leaves of *Leucaena diversifolia* were homogenized with acetone:H_2O containing 1% ascorbic acid and subsequently treated with diethyl ether and ethyl acetate. The aqueous phase was chromatographed over Sephadex LH-20 column (60x 2.5 cm) and eluted with 50% aqueous methanol. Pooled eluants were rechromatographed over Sephadex LH-20 and gradient elution methanol : Water (3:7) were furnished brown solid on lypholization. Finally it was further purified by preprative TLC on cellulose (E Merck) using Butanol : Acetic acid : Water (upper layer 4 : 1 : 5) to give compound, designated as LD (Rf = 0.72) .

STUDY OF COMPOUND LD :-

Compound LD light brown amorphous solid melted at 190 – 92^{0}C . The LD gave positive test for flavonoid (Geissman, 1955) The FABMS suggested its molecular formula $C_{44}H_{30}O_{13}$.

PRESENCE OF IMPORTANT FUNCTIONAL GROUPS IN LD :-

The IR peaks of compound LD on comparison with that of precedent literature [Silverstein *et.al.*, 1974, Cross, 1959, Finar, 1983] revealed the presence of peak at 3460 cm^{-1} hydroxyl group, 1650cm $^{-1}$ Carbonyl group, 1363 cm^{-1} gem dimethyl grouping 819,740 cm^{-1} *cis* olefinic bonding and 1510, 1166 cm^{-1} fused furan ring.

UV SPECTRUM OF LD :-

The absorption maxima displayed by LD in methanol along with various shift reagents were as follows.

252 270 35

252 270 352

6.65 6.66 6.6

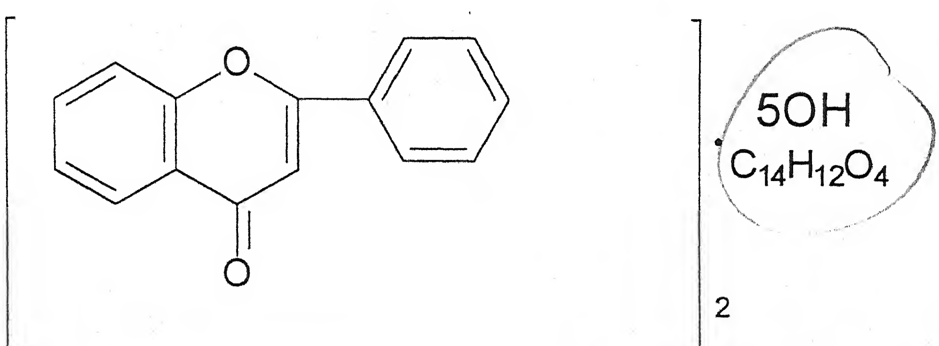
$\lambda(MeOH)_{max}$	258	270	348		
log ε	6.93	6.3	6.45		
λ $(+NaOMe)_{max}$	275	402			
log ε	6.32	6.49			
λ $(+AlCl_3)_{max}$	272	300	416		
log ε	6.21	6.06	6.47		
λ $(+AlCl_3/HCl)_{max}$	262	276	294	360	382
log ε	6.36	6.36	6.34	6.2	6.4
λ $(+NaOAc)_{max}$	268	414			
log ε	6.37	6.31			
λ $(+NaOAc/H_3BO_3)_{max}$	258	366			
log ε	6.35	6.32			

In presence of $AlCl_3/HCl$ all the bands shifted bathochromically with respect to MeOH suggesting the presence of OH at C-5 [Mabry *et.al.*, 1970]. The bathochromic shift of 54 nm in NaOMe with decreased intensity suggested OH at C-3 position of B ring [Mabry *et.al.*, 1970]. There was a hypsochromic shift of 34 nm in $AlCl_3$ relative to $AlCl_3/HCl$ indicating dihydroxylation at C-2' and C-3' [Mabry *et.al.*,1970]. It will ascribe on one of the monomeric unit by ^1H NMR during successive discussion.

BIFLAFONOIDAL NATURE OF LD :-

In the UV spectrum a greater intensity than that of corresponding monoflavone caused by weak $\pi-\pi$ conjugation as expressed by high molar extinction coefficient (log ε 6.28 and 6.4) was an indicative of dimeric nature of flavone[Jackson *et. al.*, 1971]. Further the presedence of molecular ion peak at m/z 767 $[M+H]^+$ in protonated FABMS corroborated the dimeric nature of molecule.

The aforesaid discussion led to assign the following tentative structure of LD (I)

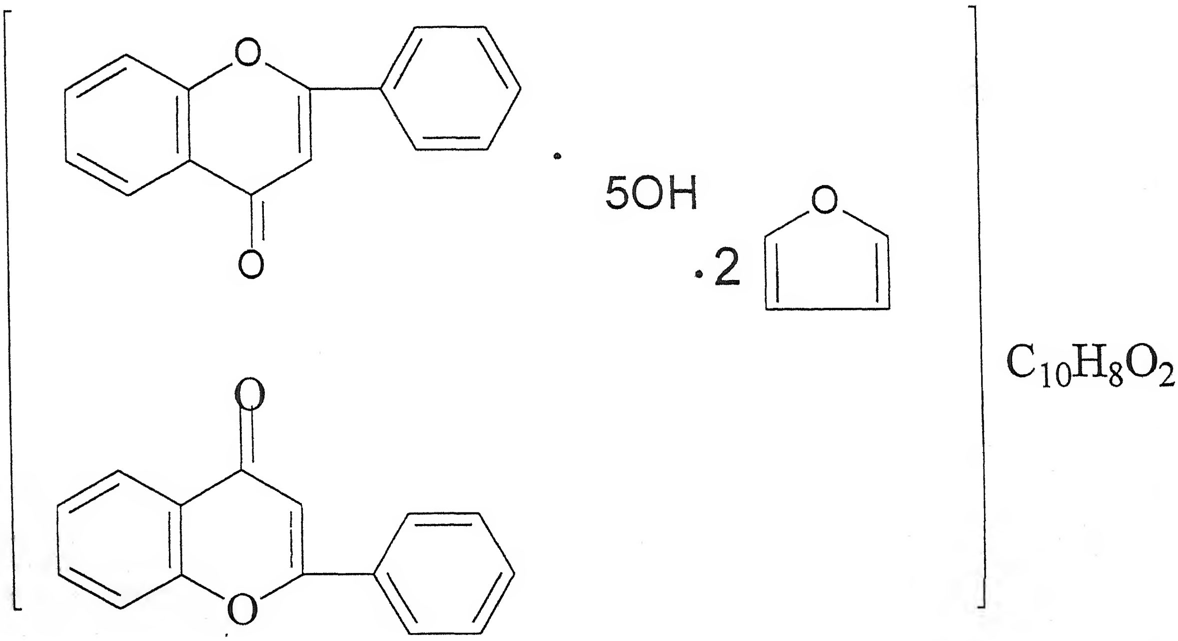


No. of C
No of H
No of O

(I)

PRESENCE OF FURAN MOIETY :-

Presence of peaks in Infrared spectrum of LD at 1570,1166 and 840 cm^{-1} suggested the presence of furan moiety in molecule [Finar, 1983]. It was also supported by the ^1H NMR of LD due to chemical shifts at δ_H 6.42 (2H,d, J = 2Hz) and δ_H 6.16 (2H, d, J = 2Hz) indicating two equally shielded furan rings [II] [Tanaka *et. al.*, 1992].

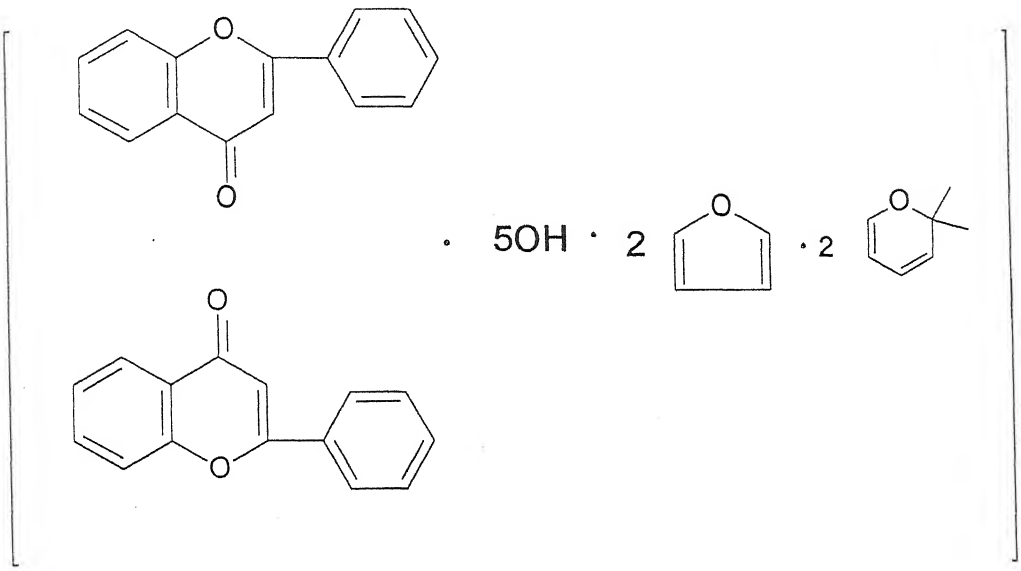


(II)

PRESENCE OF CHROMENE MOIETY :-

The IR spectrum of LD exhibited peaks at 1363,819, 740, 686 cm^{-1} for geminal diemethyl grouping and *cis* olefinic double bond of chromene ring [Conley, 1972]. Moreover ^1H NMR of LD displayed signals as high field singlet at δ_H 1.21 and δ_H 1.08 each for 6H, doublets at δ_H 7.4 (2H,d, J = 9Hz) and δ_H 6.8 [2H, d, J = 9Hz]. [Waterman and Mahmoud, 1987] which were diagnoistic

feature to the presence of two equally shielded dimethyl grouping adjacent to oxygen function and olefinic protons of chromene ring [III].



(III)

^{1}HNMR OF LD :-

The significant chemical shift obtained in the ^{1}H NMR [Fig- 1] and the structural units have been inferred with the help of evidences in literature [Wibery and Nist, 1962, Jackman, 1959] were tabulated in Table -1.

Table - 1

S. No.	Chemical Shift	Pattern	*J* value Hz	No. of Protons	Assignment
1.	6.65	s	-	2	H-3
2.	7.34	bs	-	1	H-6
3.	7.34	bs	-	2	H-4'
4.	6.42	d	2	2	H-4''
5.	6.16	d	2	2	H-5''
6.	7.4	d	9	2	H-4'''
7.	6.8	d	9	2	H-5'''
8.	8.1-9.0	Braod hump	-	1	OH at C-2'
9.	8.1-9.0	Braod hump	-	2	OH at C-3'
10.	12.5	s	-	2	OH at C-5
11.	1.21	s	-	6	CH_3 at 8'''
12.	1.08	s	-	6	CH_3 at 7'''

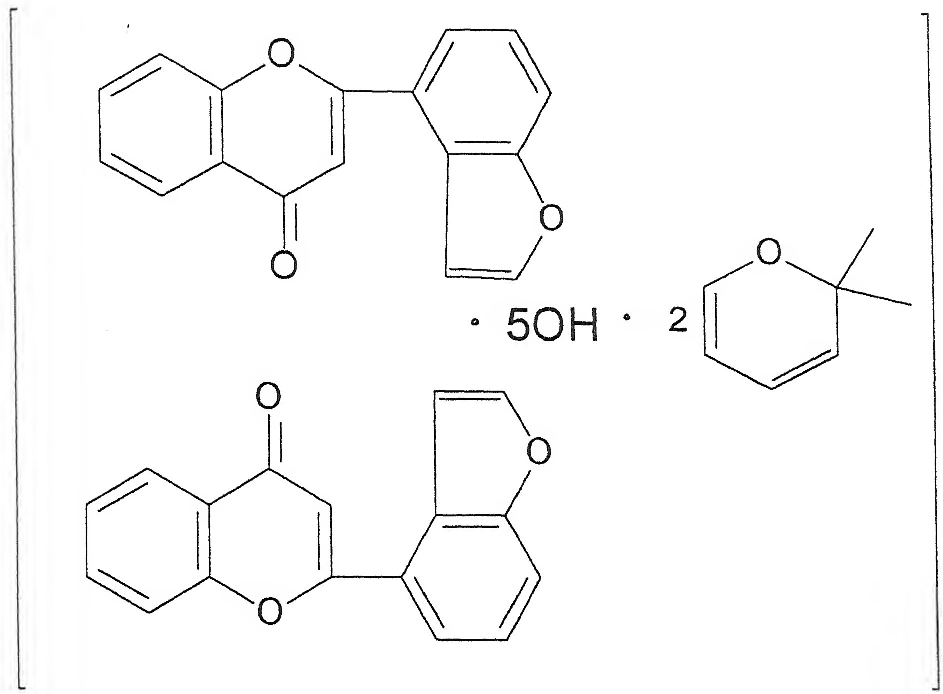
The ^1H NMR exhibited a downfield D_2O exchangeable shift in the form of sharp singlet at δ_H 12.5 integrating for two protons which was indicative of OH at C-5 [Yankep *et.al.*, 2001] and presence of a D_2O exchangeable hump between δ_H 8.1-9.0 suggested hydroxyl group [Subramanayam *et.al.*, 1977] on B ring at C-2', and C-3' of I and C-3' of II monomer. Presence of diagnoistic singlet at δ_H 6.65 for H-3 proton [Tschan *et.al.*,1996] accounting for 2H led to infer the biflavonoid structure of LD. The chemical shift appearing as broad singlet at δ_H 7.37 corresponding to 3 aromatic protons were assignable for the protons at C-6, C-4' for II monomer and C-4' of I monomer . The presence of furan and chromene moieties in ^1H NMR spectrum has already been discussed in preceeeding section.

FABMS OF LD :-

The various species assigned to fragments have been described in (Scheme -1, Fig.-2). A sharp peak at 767 $[M+H]^+$ suggested its molecular formula $C_{44}H_{30}O_{13}$. The fragmentation pattern indicated that the molecular ion m/z 593 appeared due to RDA. The yield of fragment ion peak at m/z 349 was consequence of double RDA [Drewes *et.al.*, 1967] and elimination of radicle CH = C = O, was indicative of linkage between ring A of I unit and ring B of II unit. [Roy *et.al.*,1987]. Further this was corroborated by the presence of species m/z 307 with the loss of furan moiety. The successive scissoring of interflavonyl linkage yielded molecular ion at m/z 217 [Birch *et.al.*, 1967]. The yield of fragment at m/z 709 was a resultant due, to the breaking of furan along with the loss of methyl group from chromene moiety [Reed *et.al.*,1963]. The appearence of fragment at m/z 120 revealed that it had a hydroxyl group on ring B with fused furan moiety [Nascimento *et.al.*, 1981] and the fragment m/z 166 was obtained from A ring. The yield of odd electron species at m/z 662 was resultant of the loss of chromene hydroxy with hydrogen.

POSITION OF FURAN RING :-

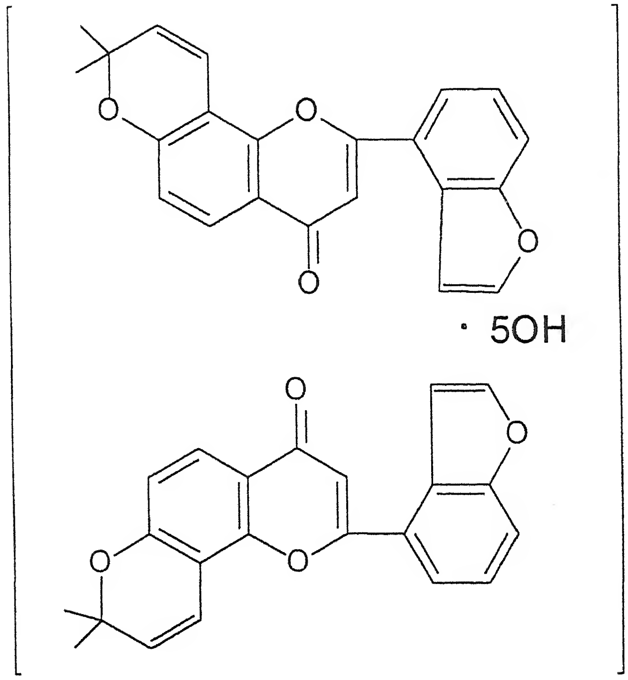
The presence of fragments at m/z 593 and m/z 307 in FABMS were indicative of fused furan ring on B ring at 5'-6' position of flavone unit, because 2' and 3' have already been assigned for OH group on page 99 and a singlet of a proton at 4'in ^{1}H NMR.(IV.)



IV.

POSITION OF CHROMENE RING: -

The molecular ions at m/z 217, 593 were indicative of chromene ring on A ring since the hydroxyl group in a ring of I unit has already been allocated at C-5 in ^1H NMR of compound LD. The chemical shift at δ_H 7.34 has also been ascribed for C-6 of I unit , C-4' of II unit leaving of C-6 (II) of one of the flavanoid unit linked to C-2' of B of I flavonoid unit. Therefore the posibility of fusion of chromene ring remained only on 7,8 position of A ring of flavanoid (V).

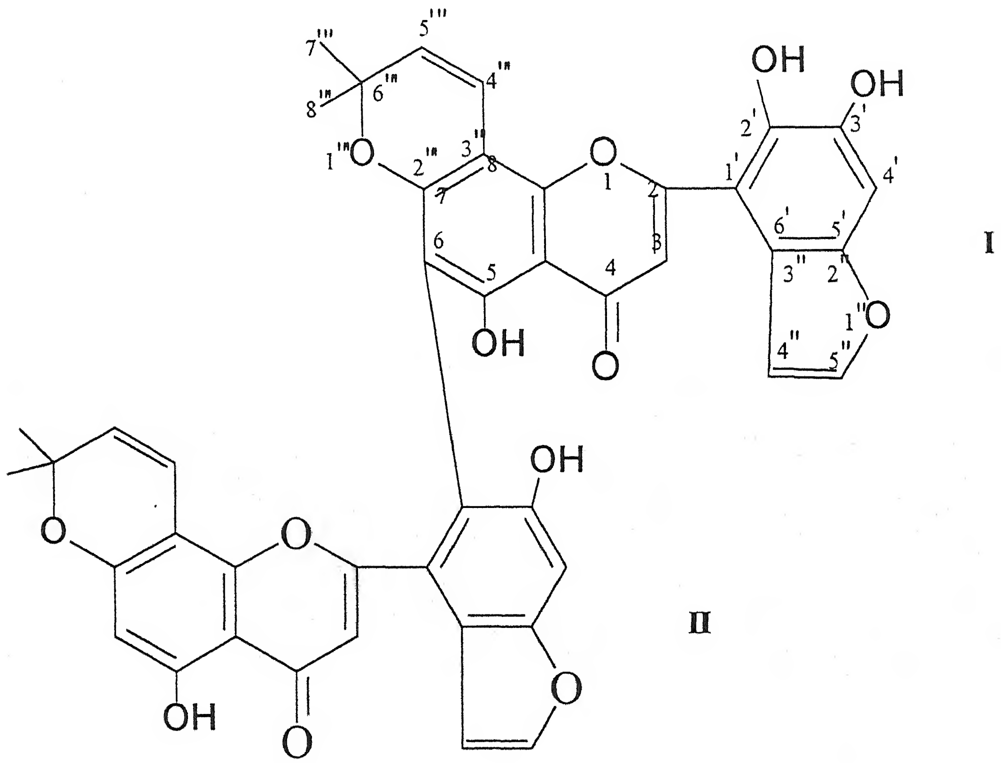


(V)

INTERFLAVONOID LINKAGE :-

The interflavonoid linkage between A ring of the unit I and B ring of unit II has already been assigned in discussion of FABMS (page 102). However on biogenetic consideration the linkage could be possible only *ortho* to the hydroxy group [Jackson *et.al.*,1971], the 2' position of B ring with OH at C-3', linked with 6 position of A ring .

On assembling of all the facts at one place led to conclude the structure of LD as **I- 2', I-3', II-3', I -5', II-5' pentahydroxy I, II [2''', 3''': 7,8] bis dimethyl chromene I, II [2'', 3'': 5', 6'] difurano, I-6- II 2' biflavonoid** (VI).



(VI)

^{1}H NMR SPECTRUM OF COMPOUND - LD

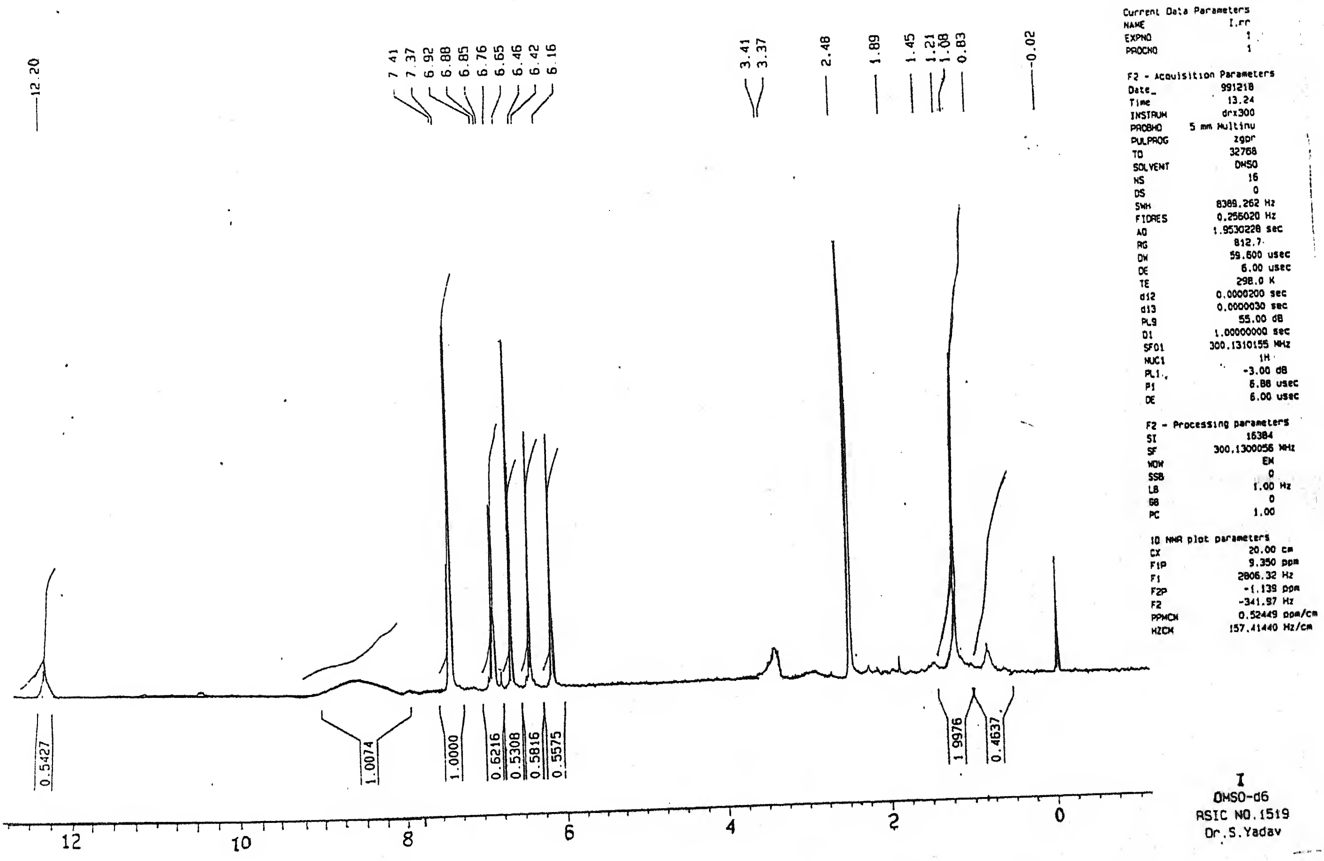


FIG - 1

FAB MASS SPECTRUM OF COMPOUND - LD

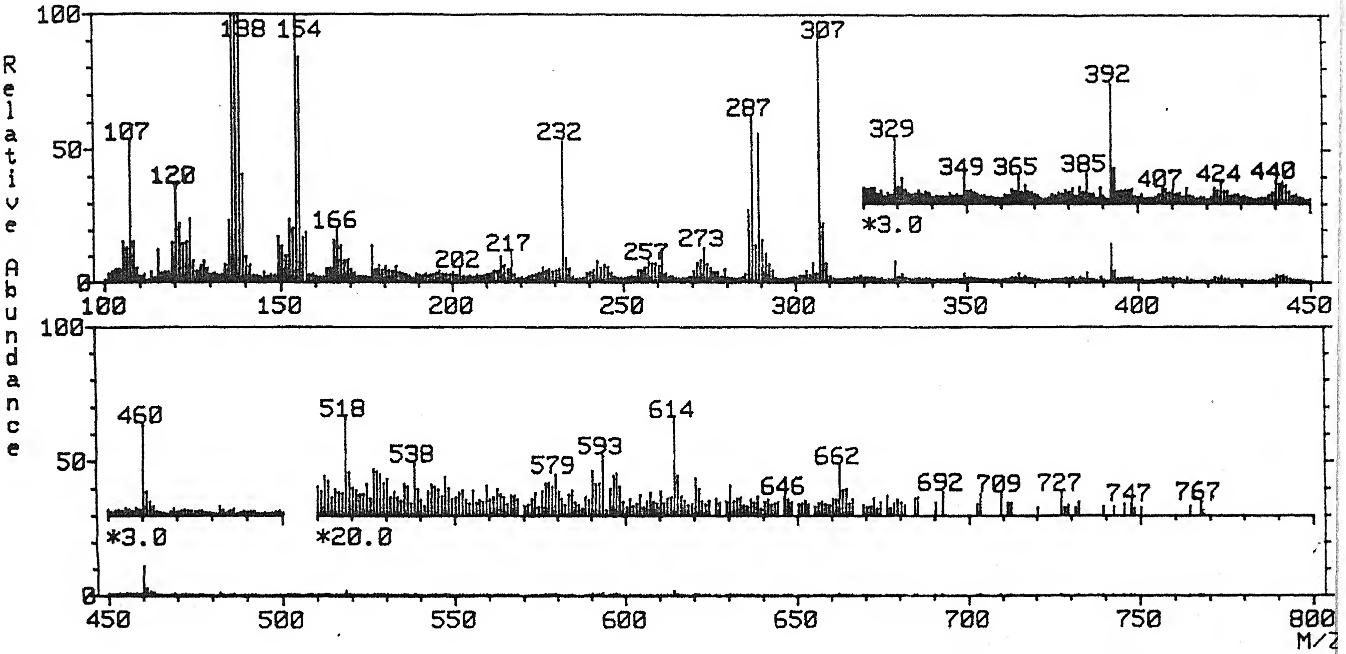
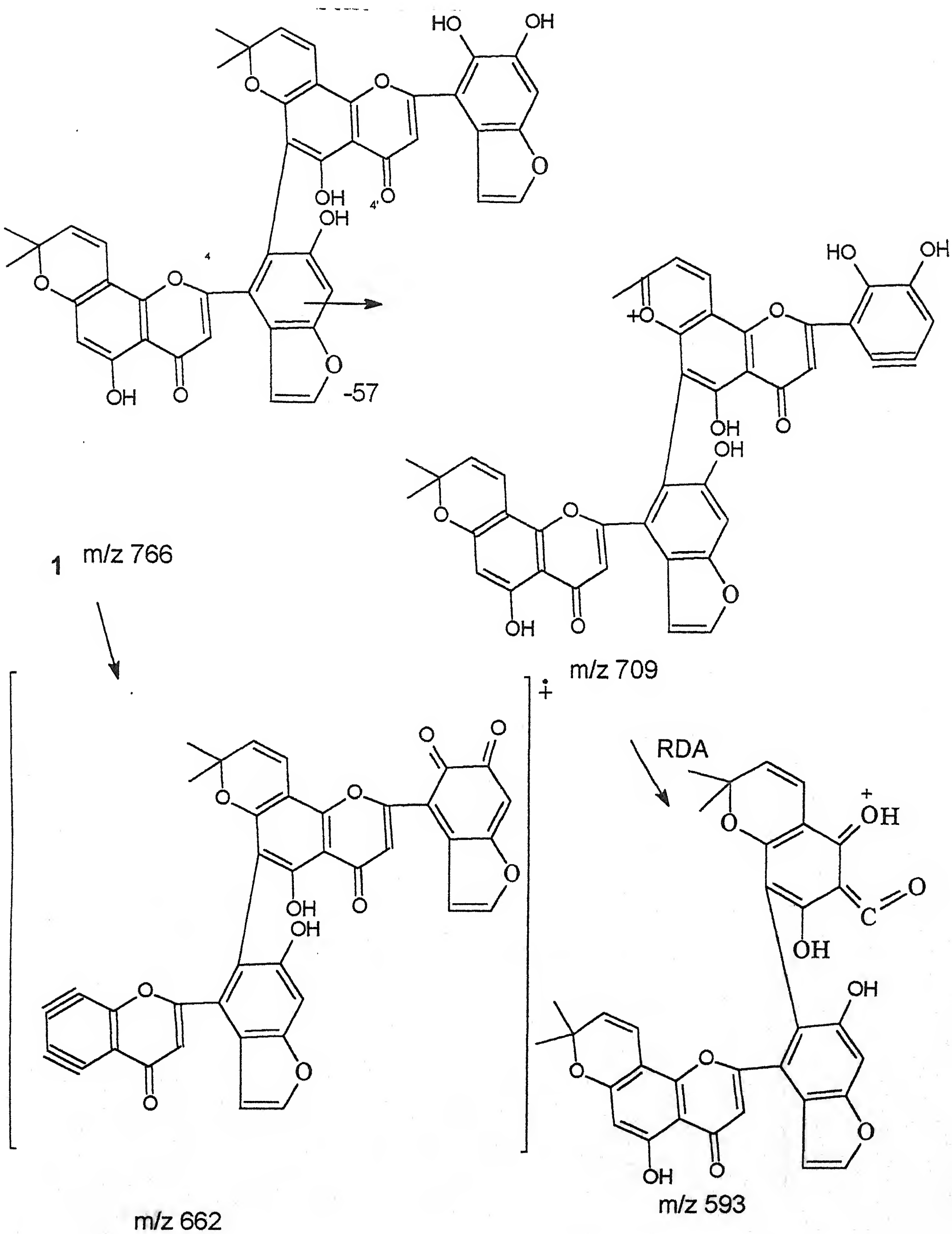


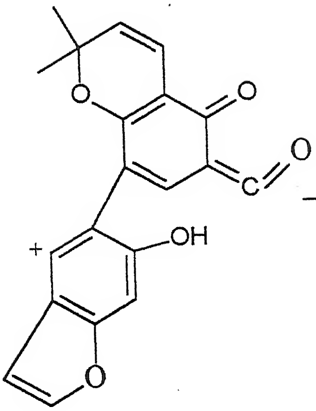
FIG - 2

SCHEME - 1

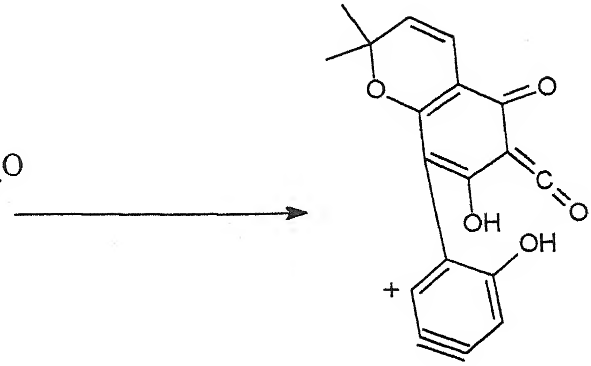


MASS SPECTRAL FRAGMENTATION OF LD

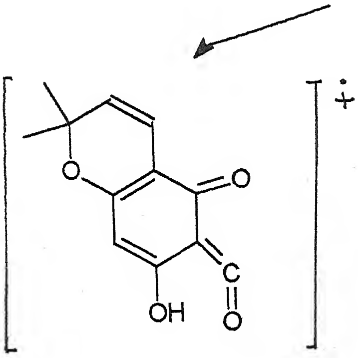
SCHEME – 1 Contd.



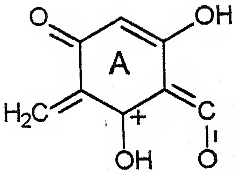
m/z 349



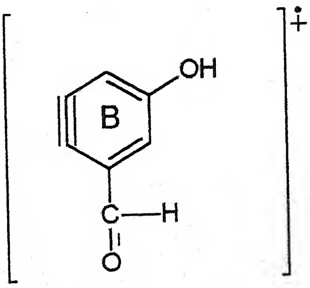
m/z 307



m/z 217



m/z165



m/z120

EXPERIMENTAL

Leucaena diversifolia leaves were collected from the CR farm of IGFRI during month of August. Fresh leaves of plants (4 kg) were homogenized with acetone: H_2O [7 : 3] containing 0.1% ascorbic acid in Bajaj blender at room temperature and filtered through buchner funnel. The filterate was reduced to aqueous phase under vacuum. The resulting aqueous phase was successively partitioned with diethyl ether and ethyl acetate. These fractions were not used for analysis . The remaining aqueous phase was subjected on rotary evaporator to eliminating traces of organic solvents and then it was diluted with methanol to 1 : 1 ratio.

It was chromatographed over already equilibrated Sephadex LH -20 column (60cm x 2.5 cm) and washed with 50% methanol till colourless washing were obtained. The pooled eluants after removal of methanol were freeze dried as dark brown solid. It was redissolved in methanol and rechromatographed on Sephadex LH-20 and eluted with methanol by increasing polarity with H_2O.

The details of eluants collected is given below in Table –2.

Table – 2

S.No	Fractions	Eluant Collected 150ml Methanol : water	Remark
1-	1 - 5	9 : 1	Light yellow colour
2-	6 - 12	8 : 2	Light yellow colour
3-	13 - 17	7 : 3	Yellow colour
4-	18 - 22	6 : 4	Light yellow colour
5-	23 - 32	5 : 5	Yellow colour
6-	33 - 37	4 : 6	Yellow colour
7-	38 - 47	3 : 7	Compound LD
8-	48 - 53	2 : 8	Yellow colour
9-	54 - 60	1 : 9	Yellow colour
10-	61 - 70	pure H_2O	Yellow colour

ISOLATION OF LD :-

The fractions 38-47 were pooled and these were concentrated under vacuum and lypholised as light brown amorphous powder (38 mg.) paper chromatography Rf (0.72) BAW [4 : 1 : 5]. Soluble in methanol, sparingly soluble in water m.p. 190 - 92^{0}C. It gave orange pink colour with Mg/HCl. Its spot on filter paper in presence of ammonia appeared as flourscent light blue in UV light. It gave molecular formula C_{44} H_{30} O_{13} $[M + H]^+$ 767 by protonated Fast atomic bombardment Mass Spectra.

INFRARED SPECTURM OF LD:-

IR ν KBr. max	3460	O-H stretching
	2910	C-H stretching
	1650	Hydrogen bonded carbonyl
	1363	Gem dimethyl grouping
	1510	furan ring
	1166	
	819	olefinic bond
	740	

FABMS OF LD:-

767, 747, 727

709, 692, 662, 646

614, 593, 579, 538

518, 460, 392, 329

307, 287, 273, 232

217, 202 166, 154

138 (BP) 120, 107

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Chapter - V

Chemical and Biochemical assessment of leaves of *Albizia procera, Bauhinia purpurea* and *Leucaena diversifolia.*

India has about 1/6th of total world livestock population with 416 million heads [Diwedi, 2000]. Livestock production is still constrained due to lack of feed. Tree leaves are important source to improve quantity and quality of available fodder particularly in dry season. The proximate analysis of leaves of *Bauhinia purpurea, Leucaenea diversifolia* and *Albizia procera* revealed that the leaves of these species were rich in crude protein as it was varying between 22-28%. The fibre part of the feed for the ruminant is very important because of the fact that it provides the energy for vital activities of body. The fibre content in leaves under investigation were determined as NDF, ADF, cellulose and hemicellulose, were in adequate quantities needed for rumination, ensalivation and ruminal buffering due to cation exchange needed for voluntary intake [Vansoest and Mason, 1991].

RESULTS

The detail description of the plant species *Albizia procera*, *Bauhinia purpurea* and *Leucaena diversifolia* have already been discussed in earlier chapters.

The proximate constitutents on dry matter basis of *Albizia procera*, *Bauhinia purpurea* and *Leucaena diversifolia* leaves have been shown in Table-1. (Fig.-1,2&3). The chemical and biochemical nature of phenolics and their different form have been presented in Table-2.

The crude protein content in *Bauhinia purpurea*, *Leucaena diversifolia* and *Albizia procera* leaves were appreciably on higher side as 28.23%, 24.86%, and 22.08 % respectively, in comparison to any leguminous herbage which normally possessed 15 to 20% [Aganga *et.al.*, 1999, Ranjhan, 1981]. The cell wall fraction in the leaves estimated as Neutral Detergent Fibre (NDF) and Acid Detergent Fibre (ADF) were found within normal range[Ranjhan, 1981, Bhadoria. *et.al.*,

2002]. Both NDF and ADF content were highest in *Albizia procera* (43.87 % and 39.02 %) followed by *Bauhinia purpurea* (43.174 % and 37.70 %) and *Leucaena diversifolia* (35.09 % and 26.81 %.). The hemicellulose content was predominated in *Leucaena diversifolia* (8.17%) following *Bauhinia purpurea* (5.46%) and *Albizia procera* (4.85%). The acid detergent lignin (ADL) in leaves was found 14.74%, 9.054% and 11.11% in *Albizia procera*, *Bauhinia purpurea* and *Leucaena diversifolia* respectively. The *invitro* dry matter digestibility (IVDMD) was observed as the lowest 38.17% in *Bauhinia purpurea* and the highest 51.59 % in *Albizia procera* followed by 50.67% in *Leucaena diversifolia*.

The evaluation of antinutritional factors as phenolics revealed that there were total phenolics (Catechin equivalent) 154.73 mg/g in *Bauhinia purpurea*, 42.53 mg/g in *Leucaena diversifolia* and 25.053 mg/g in *Albizia procera*.

The condensed tannin determined by Vanillin/HCl assay was 195.0 mg/g in *Bauhinia pupurea* 21.07 mg/g in *Leucaena diversifolia* and 16.48 mg/g in *Albizia procera* . However total proanthocyanidin percentage determined by BuOH/HCl method were 6.083 in *Bauhinia purpurea* 0.623 in *Leucaena diversifolia* and 0.253 in *Albizia procera*. The free proanthocyanidins were 14.45%, 6.55%, and 2.72% in *Bauhinia purpurea*, *Albizia procera* and *Leucaena diversifolia* respectively. Protein bound proanthocyanidins were found to be 13.304%, 0.501% and 13.148% in *Bauhinia purpurea, Albizia procera* & *Leucaena diversifolia* respectively. The maximum concentration of fibre bound proanthocyanidin were present in *Bauhinia purpurea* (5.106 %) *Leucaena diversifolia* (1.096 %) and *Albizia procera* (0.62%).

The reactivity of tannins/polymerised phenolics were determined by assessing their affinity with protein using Bovine Serum Albumin (fraction V) as test protein .The phenolics present in all the three species were able to bind protein and formed tannin protein complex. The tannin protein complex prepared from the

phenolic extract of *Bauhinia purpurea, Leucaena diversifolia,* and *Albizia procera* leaves were found to contain phenolics 6.63%, 3.01% and 2.10% whereas the *Bauhinia purpurea, Leucaena, diversifolia* and *Albizia procera* showed protein precipitating capacity as 7.43% , 0.129% and 0.694% respectively. The protein precipitable phenolics in *Leucaena diversifolia, Bauhinia purpurea* and *Albizia procera* were 51.73%, 64.94% and 41.26% respectively.The relative degree of polymerization determined as ratio of polymerized proanthocyanidin absorbed at 550 nm and 500 nm were found to be 0.322 in *Leucaena diversifolia* 1.366 in *Bauhinia purpurea* and 0.785 in *Albizia procera..*

The ratio between protein precipitating capacity and protein precepetable phenolic known as specific activity of tannins were the highest in *Bauhina purpurea* (1.12) followed by *Albizia procera* (0.33) and *Leucaena diversifolia* (0.052). The genus Leucaena is well known for a toxic non proteineous phenolic amino acid named as Leucaenol or Mimosine. *Leucaena diversifolia* was found to contain mimosine 1.711 % on DM basis

Table -1

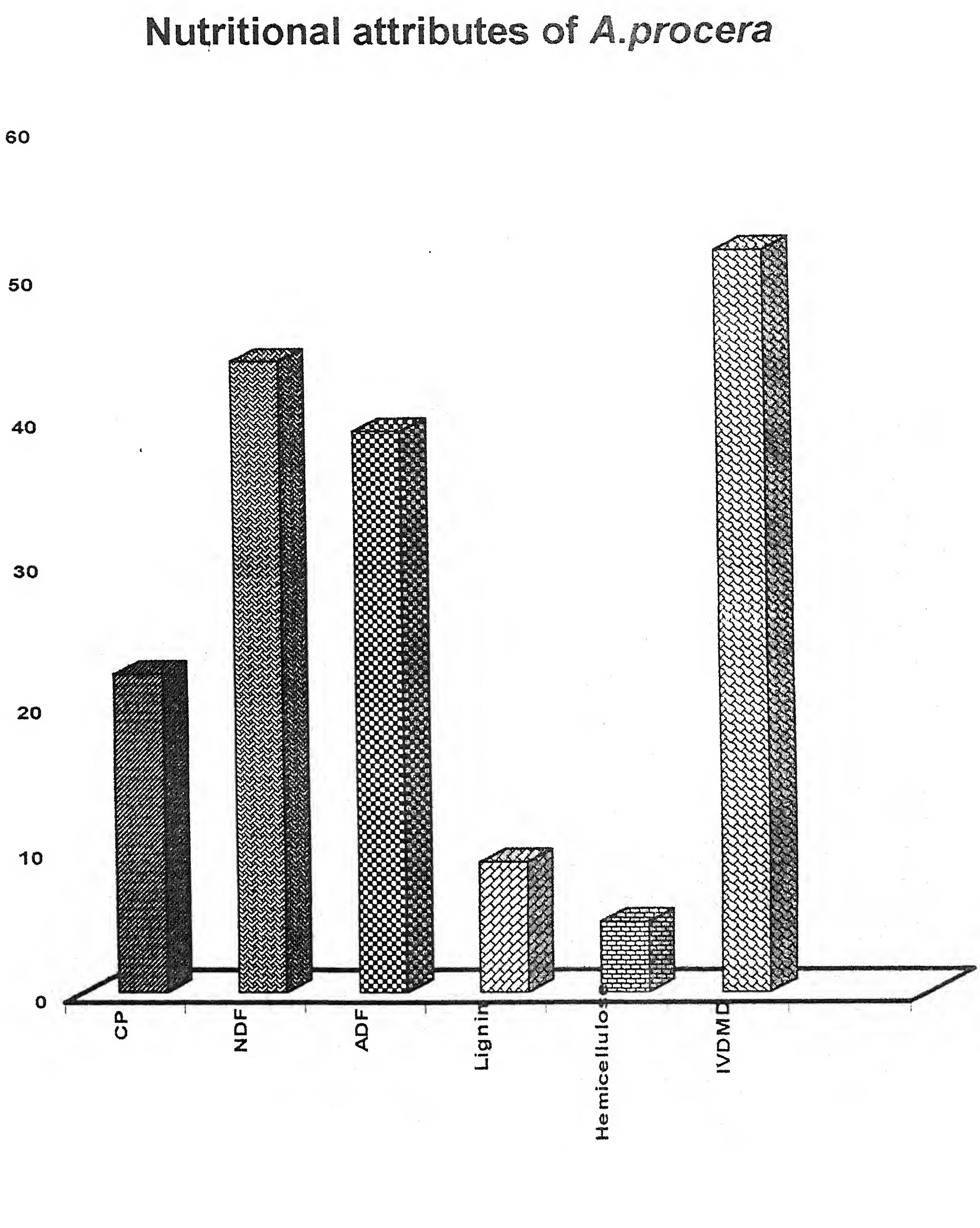
Nutritional attributes of three fodder tree leaves - in % (DM basis)

ATTRIBUTES	***L.diversifolia***	***B.purpurea***	***A. procera***
Crude protein	24.86 ± 0.377	28.23 ± 0.323	22.084 ± 0.74
NDF	35.092 ± 0.21	43.174 ± 0.421	43.872 ± 0.229
ADF	26.814 ± 0.239	37.706 ± 0.28	39.022 ± 0.232
Lignin	11.112 ± 0.264	14.74 ± 0.282	9.054 ± 0.188
Hemicellulose	8.178	5.468	4.850
IVDMD	50.676 ± 0.303	38.17 ± 0.212	51.59± 0.494

TABLE -2.

Phenolic Attribute of tree leaves

Phenolic attributes	*L.diversifolia*	*B.purpurea*	*A.procera*
CT/PA mg/g (Vanillin/HCl)	21.07± 0.37	195.0 ± 2.24	16.48 ± 0.002
CT/PA % (Butanol/HCl)	0.623 ± 0.001	6.083 ± 0.25	0.253 ± 0.001
Total phenolics mg/g (Catechin equivalent)	42.53 ± 0.23	154.73 ± 1.28	25.0532±0.001
Phenol in tannin protein complex (X) (mg/g)	3.01 ± 0.90	6.63 ± 1.21	2.10 ± 0.06
Protein in tannin protein complex (Z) mg/g	0.129 ± 0.009	7.43 ± 0.14	0.694 ± .005
Specific Activity (Z/X)	0.052 ± 0.003	1.12 ± 0.14	0.33 ± 0.025
Protein precipitable phenols. (X/Y)*100 (%)	51.73 ± 2.9	64.94 ± 2.8	41.26 ± 1.57
Relative degree of polymerisation	0.322 ± 0.005	1.366 ± 0.02	0.785 ± 0.06
Free proanthocyanidins	2.72 ± 0.09	14.45 ± 0.11	6.55 ± 0.01
Protien bound proanthocyanidins	13.148 ± 0.042	13.304 ±0.13	0.501 ±0.06
Fiber bound proanthocyanidins	1.096 ± 0.086	5.106 ±0.03	0.623 ±0.19



Nutritional attributes of *A.procera*
60
50
40
30
20
10
0
CP
NDF
ADF
Lignin
Hemicellulose
IVDMD

FIG-1

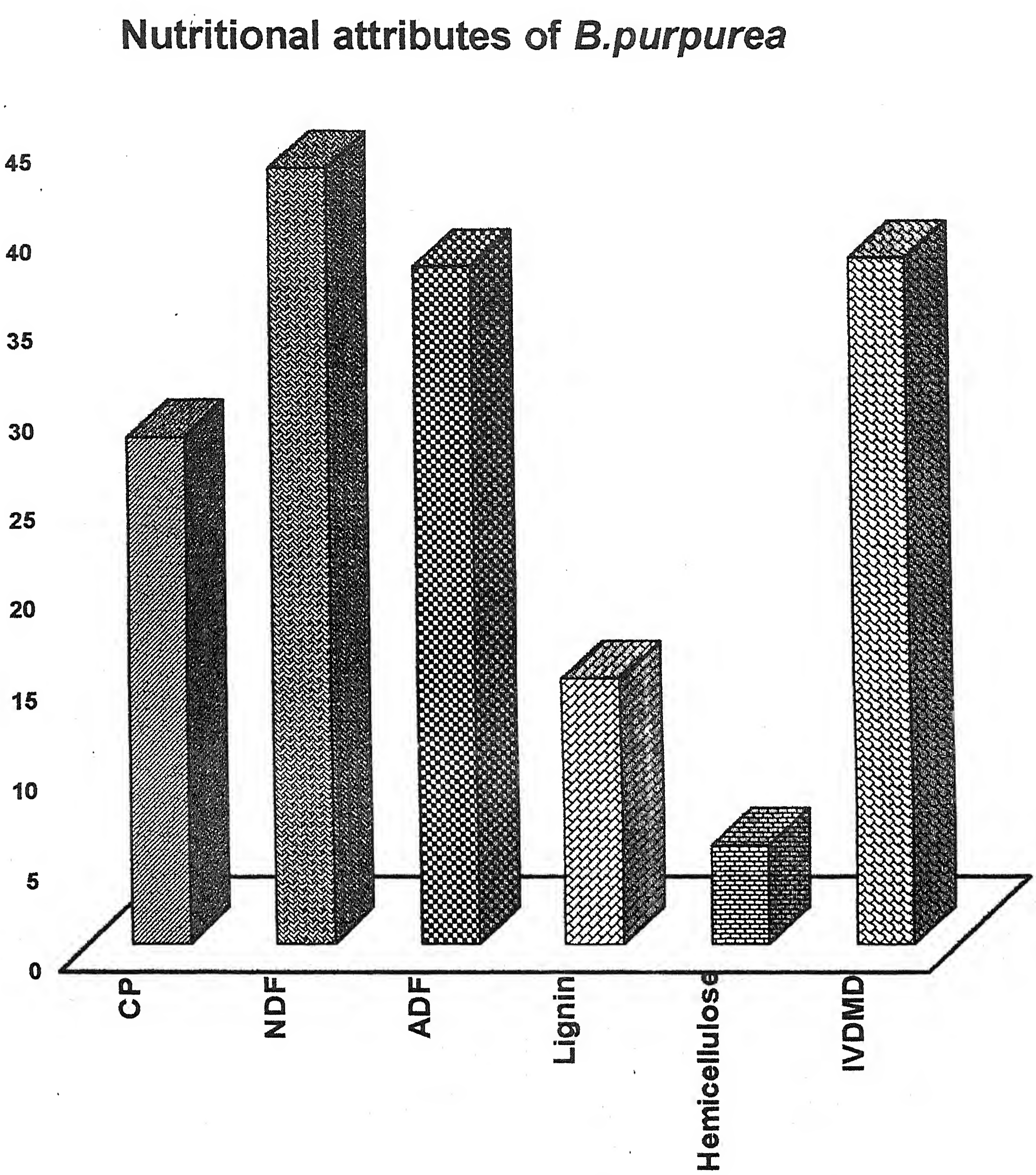
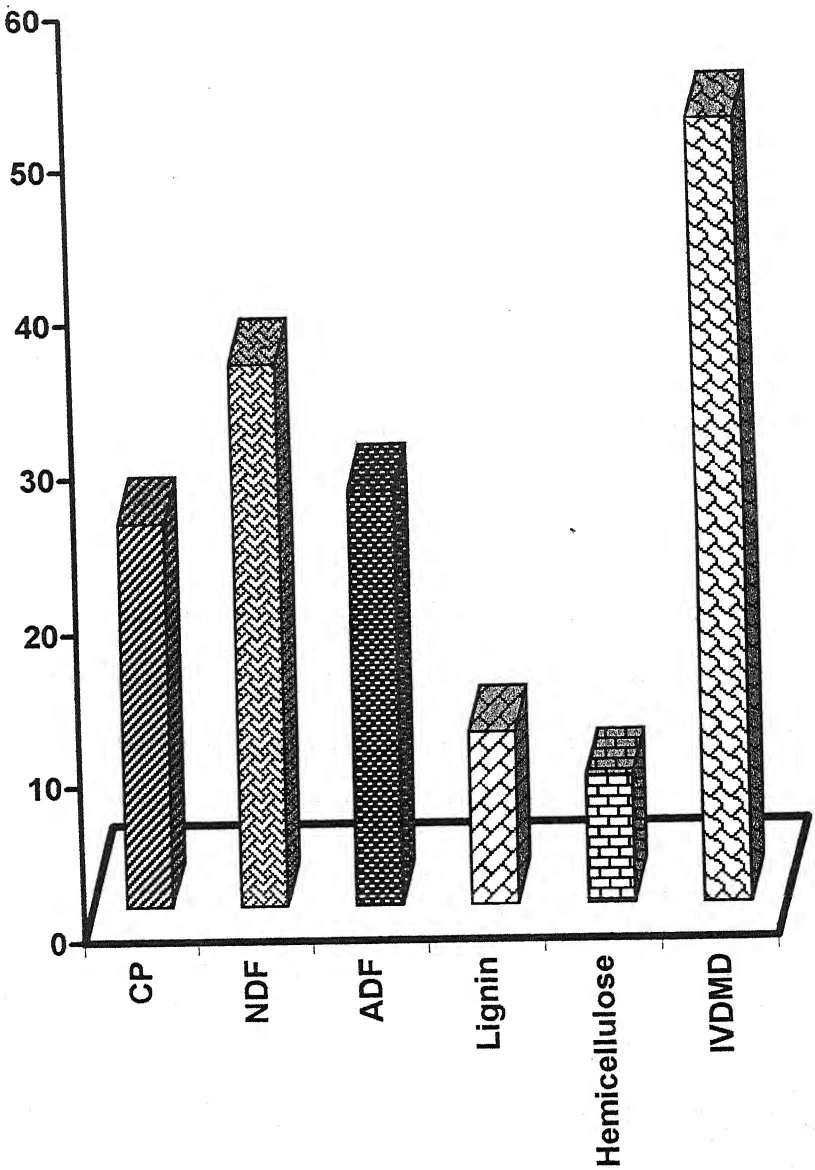

Nutritional attributes of *B.purpurea*
45
40
35
30
25
20
15
10
5
0
CP
NDF
ADF
Lignin
Hemicellulose
IVDMD

FIG 2

Nutritional attributes of *L.diversifola*



DISCUSSION

The *in vitro* dry matter digestibility of *Bauhinia purpurea* was the lowest as 38.17% while *Albizia procera* and *Leucaena diversifolia* were moderately digestible as 51.59% and 50.67% respectively. The digestibility of the feed is very much affected by the presence of lignin and flavon-3- ol, polymerised phenolics commonly referred as condensed tannin [Porter, 1994]. Lignin is highly methoxylated phenolic polymer produced by the free radical condensation of cinnamyl alcohal, *trans-p*-coumaryl alcohal, coniferyl alcohal and *trans*-sinapyl alcohal [Gordon, 1978]. A single unique structure cannot be established for lignin because of elongation of the polymer is a random process. The lignin plays significant structural role in plants and is critical factor for ruminant digestion. It is found in all the vascular plants and helps to maintain the rigidity of vascular system [Harkin, 1973]. Lignin alike other phenolics have defensive role by reducing the cell wall digestibility [Van soest, 1994]. The lignin content in *Bauhinia purpurea* , *Leucaena diversifolia* and *Albizia procera* were 14.74 % , 11.11 % and 9.054% respectively. The negative correlationship was observed between lignin content and *in vitro* dry matter digestibility of the leaves of these fodder tree as $r = 0.85$ ($P<0.05$, $n = 5$), $r = 0.201$ ($P>0.05$, $n = 5$) and $r =0.43$ ($P>0.05$, $n = 5$) in *Albizia procera, Leucaena diversifolia* and *Bauhinia purpurea* respectively. It was in agreement with earlier studies because the association of lignin with cell wall carbohydrate lowers the digestibility [Vansoest ,1994, Singh *et.al.*, 2000].

The evaluation of phenolics from the leaves as antinutritional factors showed that *Bauhinia purpurea* leaves were found to contain condensed tannin 195.0 mg/g (Catechin equivalent) (Vanillin/HCl assay) and total phenolics 154.73 mg/g catechin equivalent . Chemically the critical level of condensed tannin has

been reported to be ~5% on DM basis [Barry and Duncan, 1984, Barry, 1989] and if ingested by ruminants for 15 days continuously as sole diet leads to tannin toxicity [Kumar and Vaithynathan, 1990, Negi, 1982] manifesting sedimentation in urine, odema on mandibular region and death. *Leucaena diversifolia* and *Albizia procera* did not contain above critical level of condensed tannin and may be fed to animals as sole feed.

In *Bauhinia purpurea* there was 43.97% free proanthocyanidin of total proanthocyanidin where as in *Leucaena diversifolia* and *Albizia procera* it was 16% and 85% respectively (Fig – 4,5,& 6). The protein bound proanthocyanidin constituted 78% , 6.5% and 40% of the total proanthocyanidin in *Leucaena diversifolia*, *Albizia procera*, and *Bauhinia purpurea*. The fibre bound proanthocyanidin shared in lesser amount with maximum part in *Bauhinia purpurea* with 15.52% *Leucaena diversifolia* with 6.43% and *Albizia procera* with 8.2%. Statistical analysis exhibited significant positive correlation between free proanthocyanidin and IVDMD with correlation coefficient $r = 0.996$ ($P<0.05$, $n = 5$) in *Leucaena diversifolia* , $r = 0.773$ ($P<0.05$, $n = 5$) in *Albizia procera* except that of *Bauhinia purpurea* with negative correlationship $r = 0.33$ ($P>0.05$, $n = 5$). The negative correlationship between free proanthocyanidin and IVDMD in *Bauhinia purpurea* might be due to presence of sufficient phenolic group to cross link protein to form protein proanthocyanidin complex enough to depress the resultant IVDMD.

The depression in digestibility and palatability due to free proanthocyanidins present in plants have already been reported McLeod [1974], Jones *et.al.*, [1994]. Further Foo and Porter [1980] stated that amount of proanthocyanidin did not matter but also molecular structure. The high molecular weight of phenolics polymer are less aligned to protein molecule [Mangan , 1988].

The positive relationship between IVDMD and protein bound proanthocyanidin have been noticed in *Bauhinia purpurea* r = 0.616 ($P<0.05$, n = 5) *Leucaena diversifolia* r = 0.462 ($P>0.05$, n = 5) and in *Albizia procera* r = 0.1627 ($P>0.05$, n = 5). In these tree leaves it was noteworthy that larger part of the proanthocyanidin is bound to protein present in cells, which could be dissociated at low pH in acidic medium and is available for digestion. [Jones and Mangan 1977], All three species of tree leaves showed negative correlationship between fiber bound proanthocyanidin with *in vitro* digestibility as r = 0.844 ($P<0.05$, n = 5) *Albizia procera.* r = 0.607 ($P>0.05$, n = 5) in *Leucaena diversifolia* and r = 0.346 ($P>0.05$, n = 5) in *Bauhinia purpurea* because of the shielding complex cell wall matrices of non carbohydrate molecules like phenolic, sterol and lignin etc. [Buxton, *et.al.*, 1985, Hatifield, 1989], for providing defence against pathogens, microbial degradation and tissue strength. [Barry *et.al.*, 1986].

The significance of tannins or phenolics in plants for environment or nutrition may be better understood by studying their interaction with protein [Swain, 1979, Price and Butler, 1980, Hagerman and Butler, 1981]. The biological activity of polyphenolics is dependent on the formation of the ability of tannin to precipitate protein is often referred as astringency of tannin [Barahona *et.al.*, 1999].

The biochemical evaluation of phenolic content in the tree leaves envisaged that phenolics present in *Bauhinia purpurea* possessed highest protein precipitating capacity (PPC) 7.45 mg BSA/g leaves with 64.94% protein precipitable phenolics (PPP) whereas in *Albizia procera* the protein precipitating capacity was 0.694 mg BSA/g leaves and protein precipitable phenolics 41.26% and in *Leucaena diversifolia* there was protein precipitating capacity 0.129 mg BSA/g leaves and protein precipitable phenolics 57.73%. Highly significant positive correlation coefficient was noted between PPC and PPP in all these three

fodder tree leaves r = 0.98 (P < 0.05 , n = 5) in *Bauhinia purpurea* r = 0.93 (P<0.05 , n = 5) in *Leucaena diversifolia* and r = 0.88 (P<0.05 , n = 5) and were in good support to Makkar, *et.al.*, [1990]. The ratio of protein precipitating capacity and protein precipitating phenolics known as specific activity of tannin is an index of protein bound to per unit of phenolic molecule [Hagerman & Butler, 1989 , Makkar and Singh,1991). The results showed that specific activity of *Bauhinia purpurea* was highest 1.12 followed by *Albizia procera* 0.33 and *Leucaena diversifolia* 0.032, suggesting *Bauhinia purpurea* tannin molecule has stronger affinity than that of *Albizia procera* and *Leucaena diversifolia* .

The relative degree of polymerization (RDP) indicating the size of molecule [Butler, 1982] was found 1.35, 0.785, and 0.325 in *Bauhinia purpurea*, *Albizia procera* and *Leucaena diversifolia* respectively. In current study the inverse correlationship was observed between PPC and RDP as r = 0.273 (P>0.05, n = 5) , r = 0.706 (P<0.05, n = 5) and r = 0.582 (P>0.05 , n = 5) in *Bauhinia purpurea*, *Albizia procera* , and *Leucaena diversifolia* respectively. It was in agreement with earlier studies [Goldstein and Swain, 1963 b, Jones *et.al.*, 1976, Kumar, 1983] confirming the capacity of tannin to bind with protein was dependent on molecule size of tannin as surface of molecule was decreased with polymerization [Makkar *et.al.*, 1987b]. However the negative corelationship between total phenol and protein precipitating capacity were noticed in all the three leguminous plants which was not in agreement with Martin and Martin, [1982]. This could be due to the different nature of phenolic bound to the protein [Mangan ,1988].

There are numbers of amino acids produced by plants and all do not take part in protein synthesis. In some plants few of them become toxic on their ingestion eg. Mimosine, Indospicine and Lathyrism [Verd Court and Trump, 1969,].

The ingestion of mimosine above the certain level by the ruminants causes weight loss, alopecia, damage of liver and optic nerves, leading to death of animal. The genus Leucaena is well known for the presence of mimosine in all its vegetative plants. In *Leucaena diversifolia* leaves were found to contain mimosine 1.171% on dry matter basis which was below critical level. Therefore leaves may be utilized for animals.

Distribution of Proanthocyanidis in *A.procera*

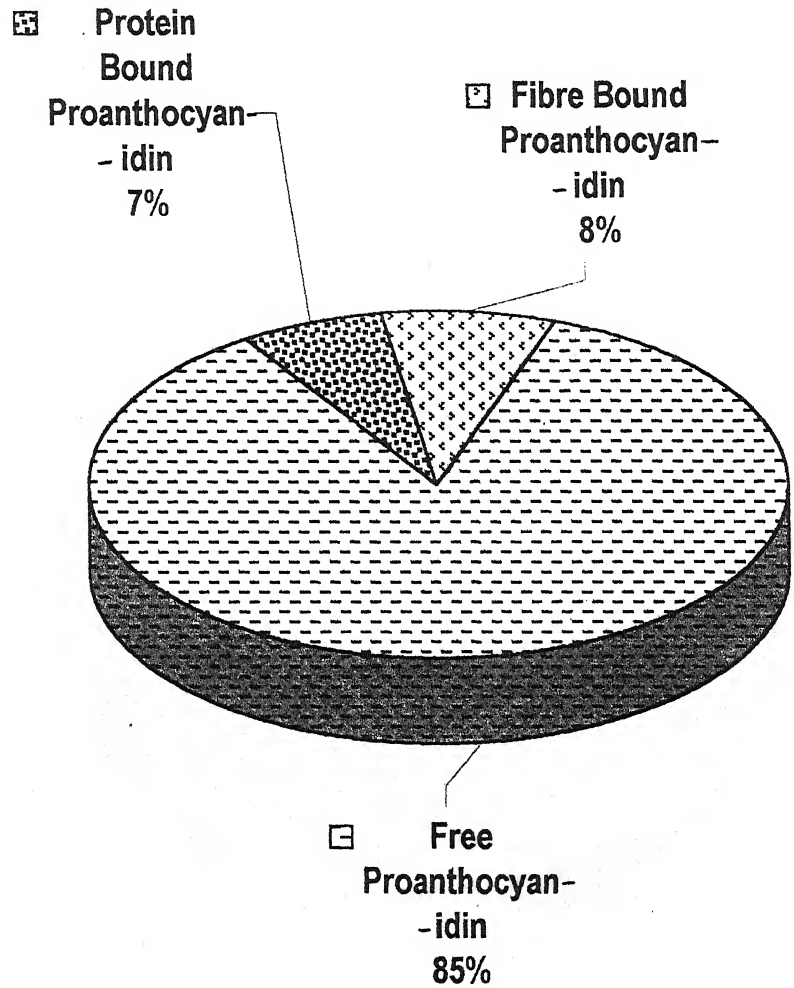


FIG - 4

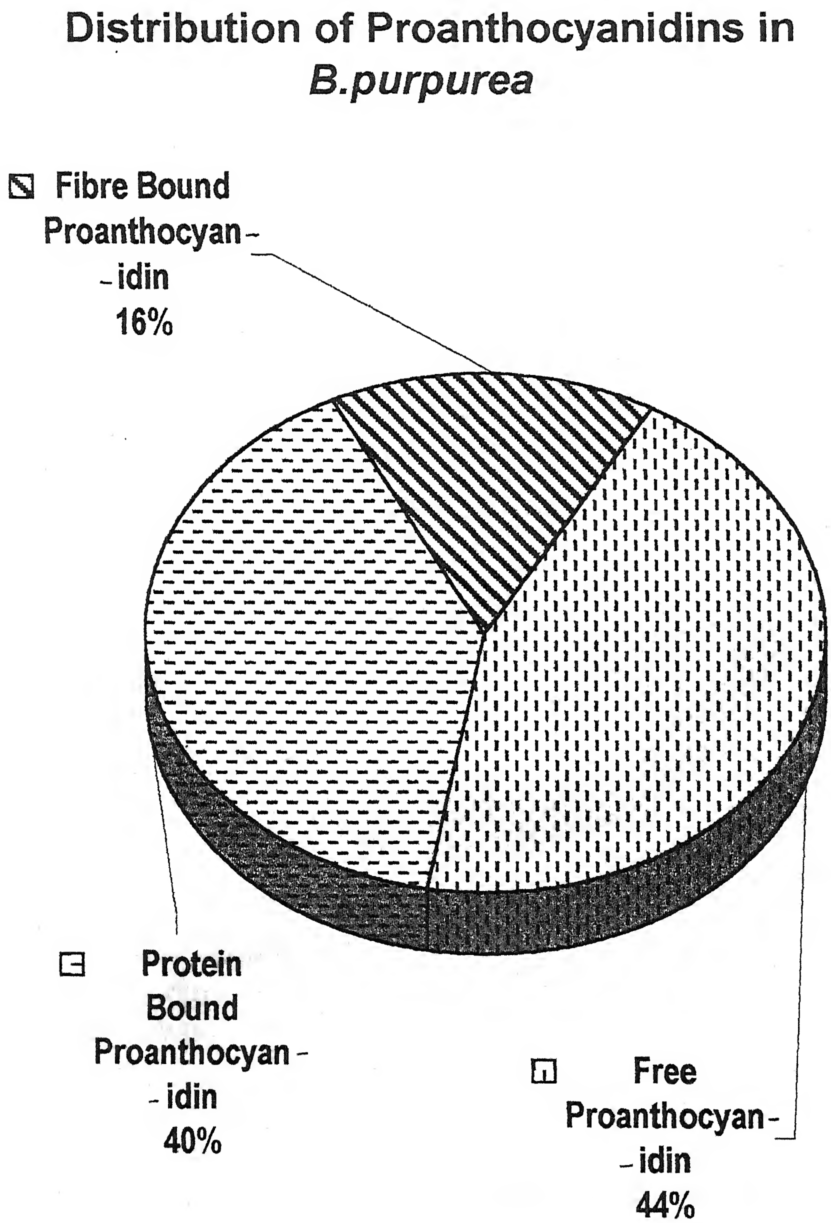

Distribution of Proanthocyanidins in
B.purpurea
Fibre Bound Proanthocyan--idin 16%
Protein Bound Proanthocyan--idin 40%
Free Proanthocyan--idin 44%

FIG - 5

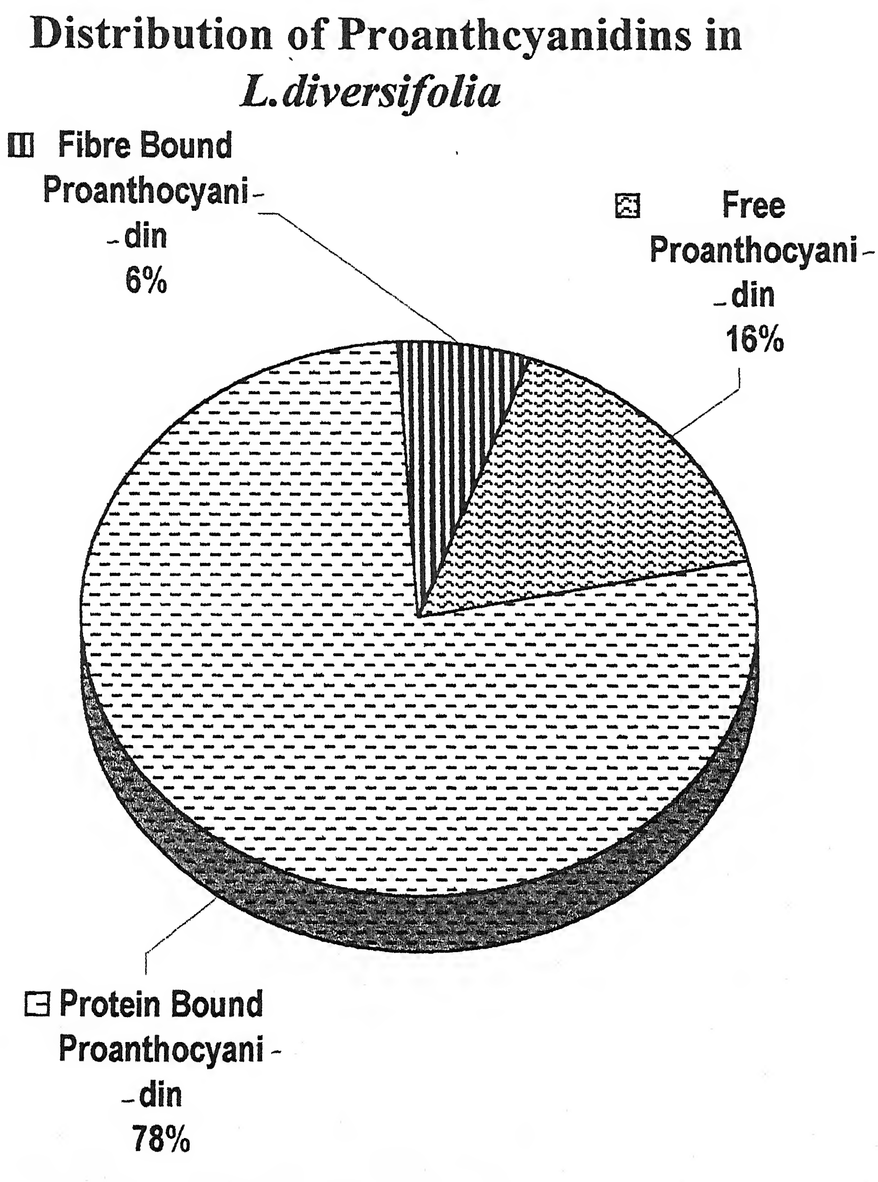

Distribution of Proanthcyanidins in
L.diversifolia
Fibre Bound Proanthocyani-din 6%
Free Proanthocyani-din 16%
Protein Bound Proanthocyani-din 78%

FIG - 6

EXPERIMENTAL

All the quantitative estimations were carried out in replicates of five. The redistilled solvents (LR) and chemicals (AR) grade were used in extraction and estimation.

SOURCE OF PLANT MATERIAL :–

The leaves of *Albizia procera* (AP) *Bauhinia purpurea* (BP) *Leucaena diversifolia* (LD) were collected after monsoon season during the year in 1998 from all side of canopy of shrubs/trees and dried in freeze drier at – 40^0C The dried leaves of each plant were separately powdered in Jaico Mill to pass 1 mm seive.

DETERMINATION OF CRUDE PROTEIN :–

The Nitrogen content of the powdered leaves were estimated by conventional Kjaldahl technique (A.O.A.C., 1990.) and multiplying the N content with factor 6.25.

CELL WALL AND ITS FRACTIONS :–

Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), Acid Detergent Lignin (ADL), Hemicellulose, were determined using the methods given by Goering and Von Soest [1970], Von Soest *et.al.,* [1991] as follows.

(i) Estimation of Neutral Detergent Fibre

Reagents –

Prepration of Neutral detergent solution: –

1- 8.61 gm Disodium ethylene diamine tetracetate dihydrate (EDTA) and 6.81 gm Sodium borate decahydrate ($Na_2B_4O_7 . 10H_2O$) were dissolved in 500 ml of distilled water by warming on boiling water bath.

2- Solution of 30 gm Sodium lauryl sulphate $CH_3(CH_2)_{11}OSO_3Na$ in 200 ml of hot distilled water.

3- 4.56 gm anhydrous Disodium hydrogen phosphate (Na_2HPO_4) dissolved in 100 ml hot distilled water.

4- Solution **1, 2** and **3** were mixed, 10 ml of 2-Ethoxy ethanol was added at room temperature to increase final volume to one litre with distilled H_2O.

5 Acetone (LR)

Method of Estimation :– 500 mg dried powdered plant leaves were refluxed with 100 ml NDF solution **4** in a 600 ml spoutless beaker for one hour and filtered through weighed sintered glass crucible. After repeated washing with H_2O and lastly with acetone, the crucible along with content was dried in a hot air oven at 100^0C and weighed till constant weight

$$\text{NDF content} = \frac{\text{Gain in wt. of crucible} \times 100}{\text{wt. of plant material} \times \text{DM \%}}$$

(ii) Estimation of Acid Detergent fibre :-

Reagents

1. **Acid detergent solution** – 20 gm Cetyl trimethyl ammonium bromide ($C_{19}H_{42}BrN$) in one litre of 1 N sulphuric acid with gradual shaking.

2. Acetone (L.R.)

Method of Estimation :-

One gm dried plant material was refluxed with 100 ml of Acid Detergent Solution in a 600 ml spoutless beaker for 1 hr. and filtered through a weighed sintered glass crucible. After several washing with hot water and finally with acetone the content was dried in the crucible in a hot air oven maintained at $100^{0}C$ for overnight, weighed till constant weight.

$$\text{ADF content} = \frac{\text{Gain in wt. of crucible x 100}}{\text{wt. of plant material x DM\%}}$$

(iii) Estimation of ADL :-

Reagents –

1. 72% w/w H_2SO_4 - Prepared by adding 583 ml con. H_2SO_4 (AR) slowly in ice cold 417 ml distilled water with constant shaking.
2. Acetone (LR) –

Estimation –

After determination of ADF the content of crucibles were covered with cooled 72% H_2SO_4 (15^0C) and kept for 3 hour. The excess of sulphuric acid was filtered off and content was washed with hot water until free from the acid. The crucible was dried in an oven at 100^0C for overnight and weighed again crucible till constant weigh (w_1). The loss in wt of crucible was corresponded to cellulose content. Then crucible was placed in a muffle furnace at 550^0C for 3 hour. The crucible was taken out in a desiccator cooled weighed again (w_1) loss in weighed was determined as lignin content.

(iv) Determination of Hemicellulose :–

The hemicellulose content in leaves were determine by subtraction of ADF content from NDF content

$$\% \text{ Hemicellulose} = \% \text{ NDF} - \% \text{ADF}$$

3. ***IN VITRO* DRY MATTER DIGESTIBILITY :- [Tilley and Terry, 1963]**

Reagents –

1. **Solution A** – 3.7gm of anhydrous Disodium Hydrogen phosphate (Na_2HPO_4) and 9.8 gm of Sodium Bicarbonate ($NaHCO_3$) in one litre of distilled water at 39^0C
2. **Solution B** – 4.7 g of Sodium chloride (NaCl), 5.7 g of Potassium chloride (KCl), 0.4 g of Calcium Chloride ($CaCl_2$), 0.6 g of Magnesium Chloride ($MgCl_2$), in 100 ml of distilled water.
3. **McDougalls buffer solution :–** 10 ml of solution B mixed to one litre solution A and maintain its pH at 6.9 by passing CO_2.
4. **Pepsin** (1: 3000 units) -

Inoculent :–

The rumen fluid for inoculation was collected before morning feeding to fistulated adult male crossbred cattle maintained on stall feeding as per usual procedure. Rumen liquour strained through muslin cloth was stored in vacuum thermos flask at 37^0C for experiment.

Method :–

In complete inert atmosphere 40 ml of McDougllas buffer solution and 10ml inoculent was added in 100 ml Erlenmayer flask containing 0.5gm dried sample fitted with a gas released valve at 37^0C. The mixed content was incubated at 39^0C for 48 hours with occasional swirling. After 48 hour

0.3 g of Pepsin and 2 ml of 6 N HCl were mixed to each flask thoroughly. The flasks were again incubated for an additional 48 hr. Finally the content was filtered through weighed sintered glass crucible and washed repeatedly with hot water and dried in hot air oven at $100^{0}C$ to constant weighed. The blank was run simultaneously.

$$IVDMD = \frac{\text{Original wt. Of sample} - \text{wt of residue} \times 100}{\text{wt of sample}}$$

1. Estimation of Total Polyphenolics [Price and Butler, 1977] :-

Reagents –

(1) 0.05 M Ferric Chloride ($FeCl_3$) in 0.01 N HCl

(2) 0.08M Potassium Ferricyanide K_3 $[Fe(CN)_6]$ in distilled water.

(3) Standard Catechin (Sigma) 2.5 mg in 50 ml of distilled water.

Preparation of Calibration curve with Catechin :–

0.5, 1ml, 2ml, 2.5 ml, 4 ml standard catechin solution were taken in duplicate and increased to 1ml by addition of H_2O. Further 3ml of reagent 1 and 2 each was added followed by 60 ml of distilled water.The OD of reaction mixture was recorded after 10 minutes at 725 nm and the calibration curve was prepared between absorbance and concentration (Fig -7).

Extraction and Estimation of Poly phenolics from Plant sample :–

200 mg of powdered leaves were extracted with 10-15 ml of methanol for 5 hours in a 50 ml stoppered conical flask. The process was repeated for 2-3 times . The

extract was filtered through glass wool and increased by methanol to 50 ml in volumetric flask.

0.1ml of plant extract with 60ml distilled H_2O was taken in 100 ml conical flask, followed by addition of 3 ml of 1 and 2 reagents and absorbance was noted at 725nm. The results were calculated from comparison with catechin calibration curve.

2. Estimation of condensed Tannin by Vanillin/HCl :-

[Broadhurst and Jones , 1978] :-

Reagents -

(1) 4 g of Vanillin in 100ml MeOH

(2) 8% HCl in MeOH

(3) Mixed solution 1 and 2 in ratio of 1:1

(4) Catechin (Sigma) – 2.5 mg in 50 ml in Methanol

Preparation of Calibration curve with Catechin :-

Catechin solutions containing 0.01,0.025,0.05,0.10, 0.125,0.25 μg/ml were prepared. 1ml of each was taken in duplicate in test tube and mixed with 5ml of reagent 3, to develop the colour and absorbance was read at 510 nm on UV/VIS spectrophotometer. The calibration curve was prepared between absorbance and concentration (Fig.- 8)

Extraction and Estimation of condensed tannin: –

500 mg plant leaves extracted with 50ml 70% acetone and volume was made up to 100 ml. in volumetric flask

1ml of extract was taken in test tube and 5ml of freshly prepared reagent 3 was added. The absorbance of developed colour was recorded at 510 nm and concentration of condensed tannin as catechin equivalent was calculated from calibration curve prepared.

Estimation of condensed tannin by BuOH/HCl [Porter *et.al.,* 1986] :-

Regents -

(1) n - BuOH/HCl reagent – 95:5 n Butanol : HCl

(2) Ferric reagents – 2% Ferric ammonium sulphate ($FeSO_4(NH_4)_2SO_4 . 6H_2O$) in 2N HCl

Method –

0.5 ml Plant extract as prepared above was taken in 10cm x 1.2cm test tube and 3ml of n - Butanol / HCl reagent and 0.1 ml of ferric reagent were added. The reaction mixture after gentle shaking was kept on boiling water bath for 75-80 minutes. As the pink colour devlopes the reaction mixture is cooled at 0^0C and the absorbance of colour was recorded at 550 nm against a blank prepared without plant extract.

$$\% \text{ Proanthocyanidins} = \frac{\text{Absorbance at 550 nm} \times 78.26}{\% \text{ DM}}$$

(4) **Estimation of free and Bound Proanthocyanidin [Terril *et.al.,* 1992] :-**

Reagents –

(1) (4.7: 2.0 : 3.3) Acetone :water : diethyl ether containing 0.1% ascorbic acid.

(2) Sodium dodecyl sulphate (SDS) and 0.5% 2- mercapto ethanol ($HS\ CH_2\ CH_2OH$) in 10 ml tris/chloride, adjusted to pH 8 with HCl.

(3) n - BuOH /HCl Reagent – 95:5 n -Butanol : HCl

(4) Ferric reagent – 2% of Ferrous ammonium sulphate $FeSO_4 (NH_4)_2 SO_4 \cdot 6H_2O$ in 2M HCl

Extraction of free proanthocyonidin :–

500mg of powdered leaves were extracted with solvent mixture 3 for 4 hours. The extractive was partitioned in separating funnel. The aqueous layer was removed quantitatively and evaporated in rotatory evaporator to remove traces of organic solvents. Further aqueous layer was centrifuged at 27000*g* remove any non tannin particulates. The supernatant was made to 100 ml in volumetric flask with water.

Extraction of protein bound proanthocyanidin :–

The residue obtained after removal of free proanthocyanidin residue was further extracted with 15-20 ml hot SDS 2-3 times. The combined extract was centrifuged for 15 min at the speed of 30000*g* and the supernatant was increased to 100 ml by SDS solution.

Extraction of fibre bound proanthocyanidins :-

It was estimated directly from the last left residue after protein bound extraction.

Estimation of Extractable proanthocyanidin-

1ml aliquot mixed with 5ml of freshly prepared Butanol/ HCl in a centrifuge tube and shaked gentaly. The tubes were placed on a boiling water bath for 75minutes followed by rapid cooling on Ice bath. Then the absorbance was recorded on a spectrophotometer at 550 nm. to calculate the extractable proanthocyanidin.

$$\% \text{ Proanthocyanidin} = \frac{78.26 \text{ x Absorbance at 550nm}}{\square\%\text{DM}}$$

Estimation of protein bound proanthocyanidin :-

1ml aliquot was taking and after adding 6 ml Butanol : HCl (95:5) was used as above

Estimation of fibre bound proanthocyanidin :-

After extraction of free and protein bound proanthocyanidin the remaining residue was used for determination of fibre bound proanthocyanidin directly by adding 30 ml of Butanol/HCl and 3 ml of SDS soultion in Erlenmayer flask. The flask were placed on a boiling water bath for 75min, cooled on ice bath and centrifuged for 15 min to pellet the solid and read absorbance on a spectrophotometer at 550 nm.

1. Biochemical Evaluation of Phenolics: -

Reagents –

(1) Standard Bovine Serum Albumin fraction V (BSA) (E Merck) 1mg/ml in 0.2m acetate buffer containing (0.17M sodium chloride and adjusted to pH 5 with NaOH solution.)

(2) Sodium Dodecyl Sulphate (SDS) Solution - 1% w/v in distilled water.

(3) Sodium Dodecyl Sulphate Triethanolamine solution - 1% SDS (w/v) in 7% (v/v) triethanolamine (TEA) in distilled water.

(4) 0.01M Ferric chloride reagent in 0.1M HCl

(5) 13.5N NaOH - Mix 54gm of NaOH in approximately 80 ml of distilled water and make the volume to 100 ml with distilled water.

(6) Glacial acetic acid

(7) BSA solution – (1mg/ml) in distilled water.

(8) Acetate Buffer – 0.20 M acetic acid containing 0.17M sodium chloride (NaCl) and adjusted to pH 4.8 to 4.9 with 2 M Sodium hydroxide(NaOH).

(9) Ninhydrin Reagent – Solution containing 4 gm ninhydrin in 100 ml 2-methoxy ethanol was mixed with 200ml of solution having 0.16 gm stannous chloride ($SnCl_2 . 2H_2O$) in 100 ml of acetate buffer and 100 ml distilled water.

(10) Tannic acid (E Merck) in SDS solution

Prepration of calibration curve :–

Tannic acid solution containing 0.001, 0.003, 0.005, 0.01, 0.02 mg/ml were prepared in SDS solution taken and volume were increased to 1ml with SDS solution following addition of 3ml SDS-TEA and 1ml $FeCl_3$ were mixed. The absorbance at 510 nm was recorded after 15-20 minutes and the calibration curve was drawn (Fig - 9)

Calibration curve with BSA for protein estimation :– Standard BSA solution were taken in duplicate, containing 50µg, 100 µg, 150 µg, 250 µg, 350 µg,. The test tubes were dried in an oven at $100^0C \pm 10$. To dried BSA in each tube and 0.6ml of 13.5 N NaOH was added. The tubes were again placed in hot air oven at $120^0C \pm 2$ for 20 min. The tubes were cooled and 0.5 ml of glacial acetic acid was added very slowly to neutralize the alkali. To this one ml of ninhydrin reagent was mixed and resulting mixture heated on a boiling water bath for 20 min. The tubes were cooled and diluted with 5 ml of distilled water and read the absorbance at 570 nm. and calibration curve was drawn (Fig-10)

Extraction of tannin [Martin and Martin, 1982] :–

200mg of ground laeves were extracted with 10ml of 50% methanol. The process was repeated for 2 to 3 times and the fixed volume was made up with 50% methanol.

Prepration of tannin protein complex [Hagerman and Butler, 1978] :–

Two ml standard 1mg/ml BSA solution and 1ml. of prepared extract were mixed in centrifuge tubes and after through swirling kept in refrigerator for 24 hour. The resulting precipitate of tannin protein complex was centrifuged at 50000g for 15 minutes to separate supernatant. The supernatant was discarded. The pellet obtained was washed 2-3 times with acetate buffer to remove unreacted protein. Later on the pellate was dissolved in 1.5ml of 1% SDS solution for furthur estimation.

Estimation of protein in Tannin Protein Complex :-

[Makkar *et.al.*, 1987 b]

0.1ml of the dissolved TPC complex was taken in a test tube and dried at 100^0 ± 5 . Then dried aliquot were hydrolysed by adding 0.6ml 13.5 NaOH at 100^0C for 20 minutes. The hydrolysed product was neutralized with 0.5ml glacial acetic acid. One ml ninhydrin reagent was then added and the resulting mixture heated in a boiling water bath for twenty minute. The tubes were cooled and 10 ml of distilled water was added .The absorbance was read at 570 nm.

Calculation of Specific Activity [Hagerman and Butler, 1989] :-

Specific activity of tannin was calculated by the ratio of protein precipitating capacity to protein precipitable phenolics.

$$\text{Specific activity of tannin} = \frac{\text{Protein Precipitating Capacity)}}{\text{Protein Precipitable Phenolics.}}$$

Determination of Relative degree of Polymerisation [Butler, 1982] :–

Reagents :–

(1) 70% Acetone in water

(2) Glacial acetic acid

(3) n - Butanol : HCl (95:5)

(4) Ferric reagent (2% Ferric Ammonium Sulphate in 0.1M HCl .)

(5) Methanol LR

(6) 0.5% Vanillin in glacial acetic acid

(7) 4% HCl in glacial acetic acid

Method :-

(i) 100mg of powdered leaves sample was extracted with 20 ml 70% acetone for 4hrs. and increased the volume of extract to 25ml in volumeteric flask, 0.5ml of extract was mixed with 3ml BuOH/HCl reagent and 1ml Ferric reagent. The tubes containing reaction mixture were placed on water bath for 1hour and cooled. After the colour development the absorbance was read at 550nm.

100mg of leaves sample were extracted for 4hours with 20ml glacial acetic acid and volume was increased to 25ml by methanol 1ml of extract was mixed with 5ml. Vanillin in glacial acetic acid + HCl in glacial acetic acid (1:1) after development of colour absorbance was read at 510nm.

$$\frac{\text{(Terminal flavan 3-ol)}}{\text{(Total flavan 3-ol)}} = \frac{\text{(Proanthocyanidine at 550 nm)}}{\text{(Proanthocyanidine at 510 nm)}}$$

Determination of Mimosine in *Leucaena diversifolia* :-

[Brewbacker and Kaye, 1981]:-

Reagents –

(1) 0.1 N HCl

(2) Activated Charcoal

(3) EDTA 1gm in 4 litre H_2O

(4) Ferric Chloride solution – 4gm in 500ml 0.1 HCl

(5) Mimosine (Sigma) - .25 mg in 50 ml 0.1 N HCl.

Procedure – One gm representative fresh sample of *Leucaena diversifolia* leaves were macerated with 9ml of 0.1NHCl in pestle mortor. Macerated material was centrifuged for 5minutes at 2000 r.p.m. One ml of supernatant was taken with 9ml of 0.1NHCl with 1.5gm activated charcoal and boiled for 15 minutes on boiling water bath. The content was filtered through Whatman No 42. filter paper. 2ml of filtrate is taken and mixed with 5ml of EDTA solution and 1ml ferric chloride solution . After 10 minutes the absorbance is read at 535 nm . A standard curve is prepared by using solution between 0.005mg. to 0.02 mimosine in 0.1 N HCl (Fig - 11).

Statistical Analysis :-

All the statistical analysis were carried out by using MS Excel software in Window 98.

Standard curve with catechin for Total Phenolics (Prussian blue method)

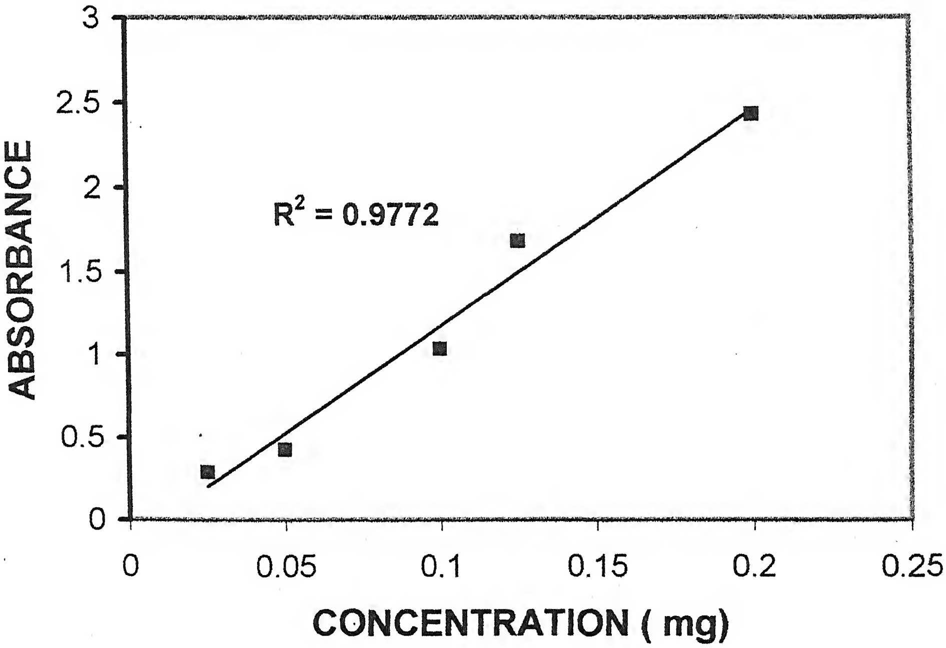


FIG - 7

Standard curve with catechin for condensed tannin (Vanillin /HCl) method

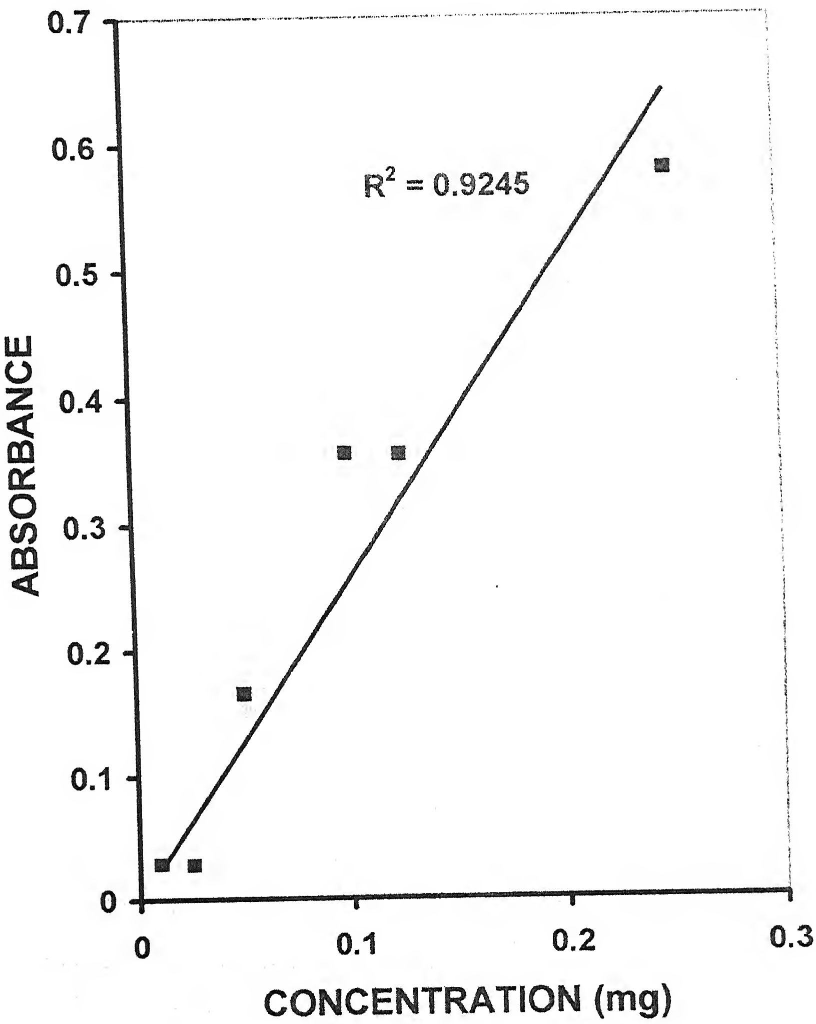


FIG - 8

Standard curve forTotal phenol
by SDS method

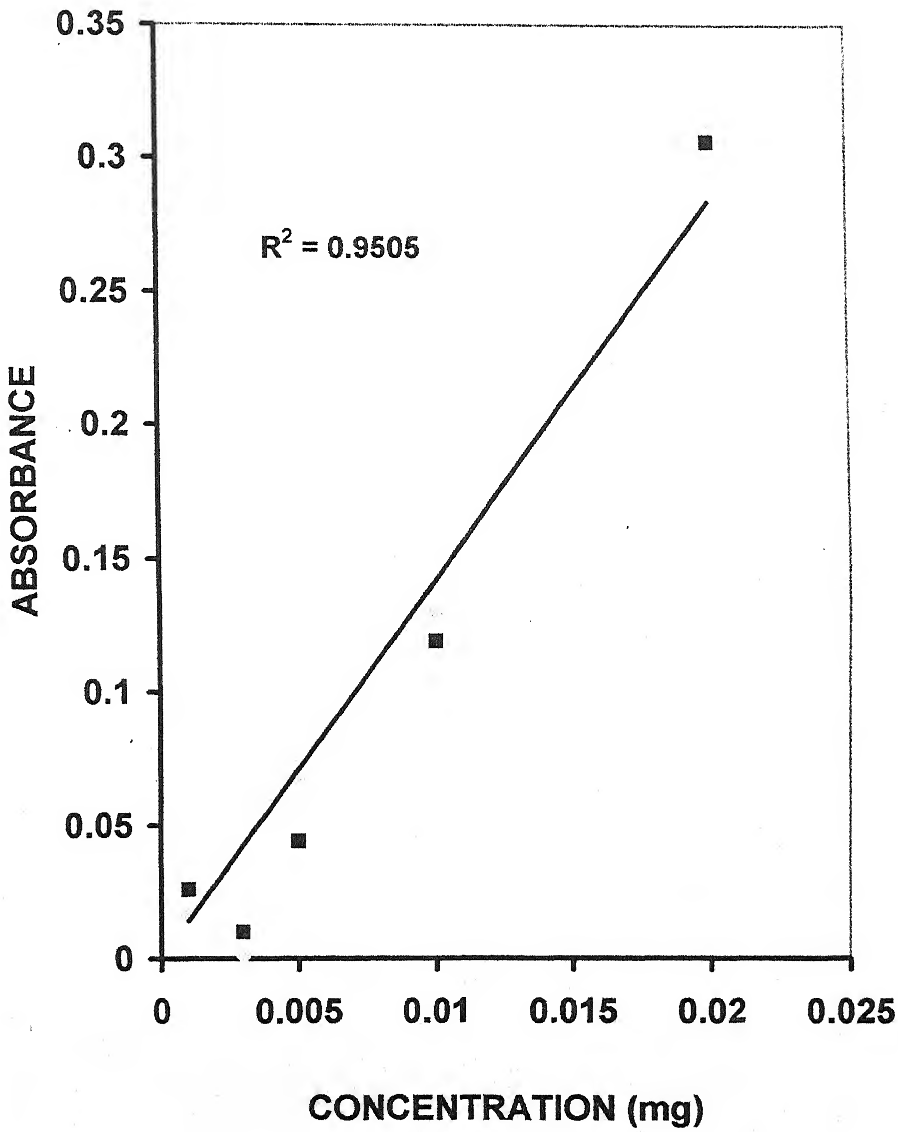


FIG - 9

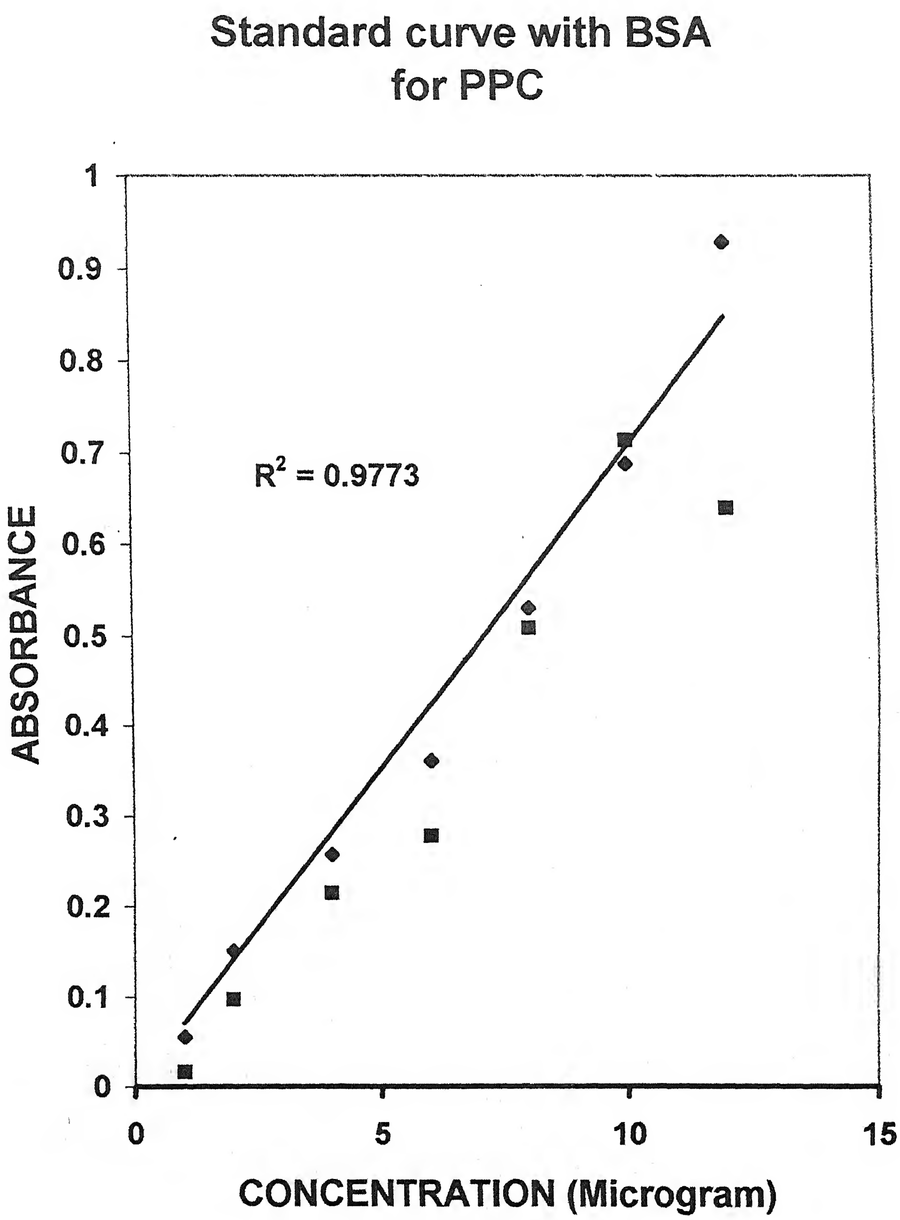

Standard curve with BSA
for PPC
$R^2 = 0.9773$
ABSORBANCE
CONCENTRATION (Microgram)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
5
10
15

FIG – 10

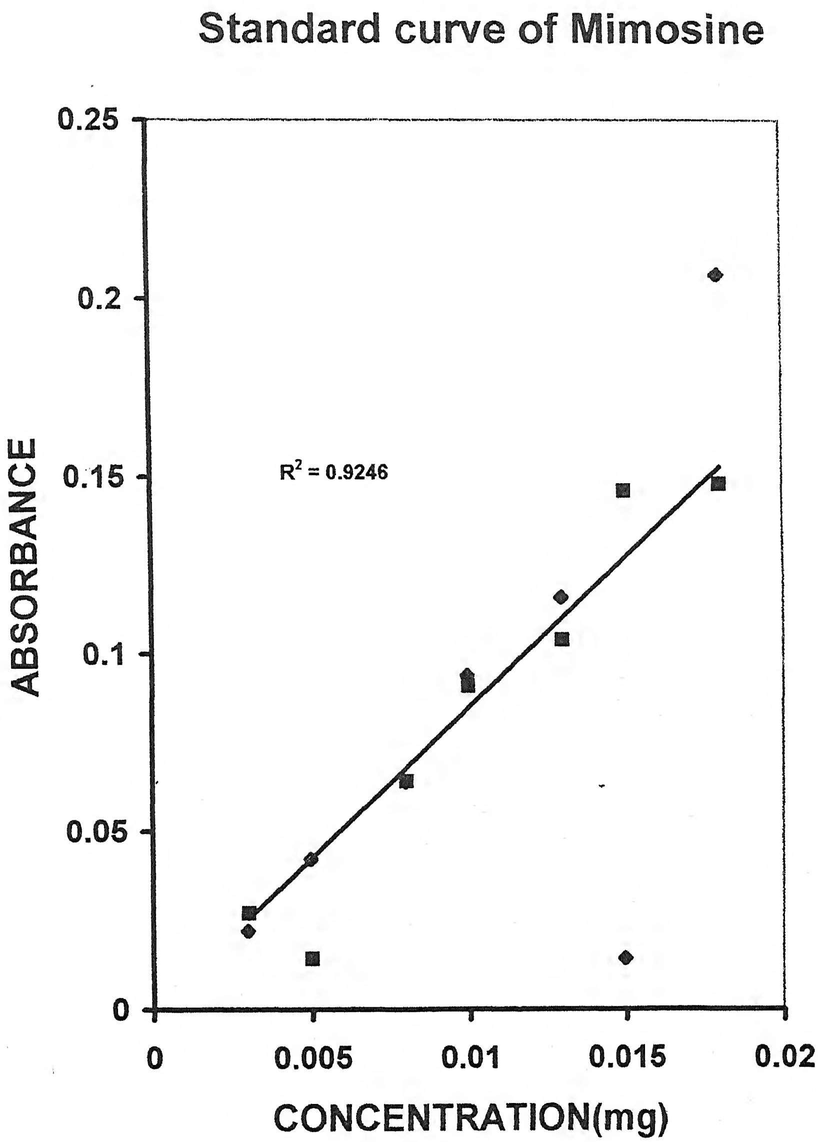

Standard curve of Mimosine
0.25
0.2
0.15
0.1
0.05
0
ABSORBANCE
R2 = 0.9246
0
0.005
0.01
0.015
0.02
CONCENTRATION(mg)

FIG – 11

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Chapter - VI

Protein Binding effeciency of isolated biflavanoid from *Albizia procera, Bauhinia purpurea* and *Leucaena diversifolia*.

The chemical reactivity of the polyphenolics and their affinity with protein is of great significance for the ecologist [Feeny, 1968, Batzli, 1983, Waterman, *et. al.*,1984, Schultz and Baldwin, 1982], nutritionist [Glick and Joslyn, 1970 a,b , Jambunathan and Mertz, 1973, Kumar and Singh , 1984], Mitaru *et.al.*, 1984), agronomist [Tipton *et.al.*, 1970, Harris and Burn , 1970. Mishra *et.al.*, 1980] and range managers [Provenza and Malechek, 1984, Robbins *et.al*, 1987]. The efficacy of polyphenol binding to protein derives in large part from the fact that polyphenols are polydentate ligands, able to bind simultaneously at more than one point to the protein structure [Hasalam *et.al.*, 1999]. High resolution NMR studies [Baxter, *et.al.*, 1997, Murray *et.al.*, 1994] generally confirm that the aromatic nucli of polyphenols provide the principal sites for association with protein. Polyphenolics protein interaction is a two stage process, first soluble complexes are formed and in second stage these complexes aggregate and precipitate (Fig-1).

The speciality of flavonoids lies not only in their polyphenolic character and the range of moleuclar weight they possess but also in their ability to complex strongly with protein [Ozawa *et.al.*, 1987). Studies of protein binding capacity of polyphenolics are not only of intrinsic scientific interest but they are also of considerable practical importance, as the taste, palatability [Joslyn and Goldstein 1964] and nutritional value [Butler and Price,1980, Kumar and Singh,1984],the pharmacological and physiological effects of plant material [Singleton, 1981] and their microbial decomposition [Bloomfield, 1957, Handley, 1961] are all properties which are substantially modified by the presence of polyphenols which they contain.

Williams [1962] found that simple phenols had little inhibitory effect on astringency of tannins increases progressively from molecular weight 576 to 1134.

enzyme and a minimum molecular weight of 500 or more appeared to be necessary for inhibitory activity. Further Bate and Smith [1973] corroborated

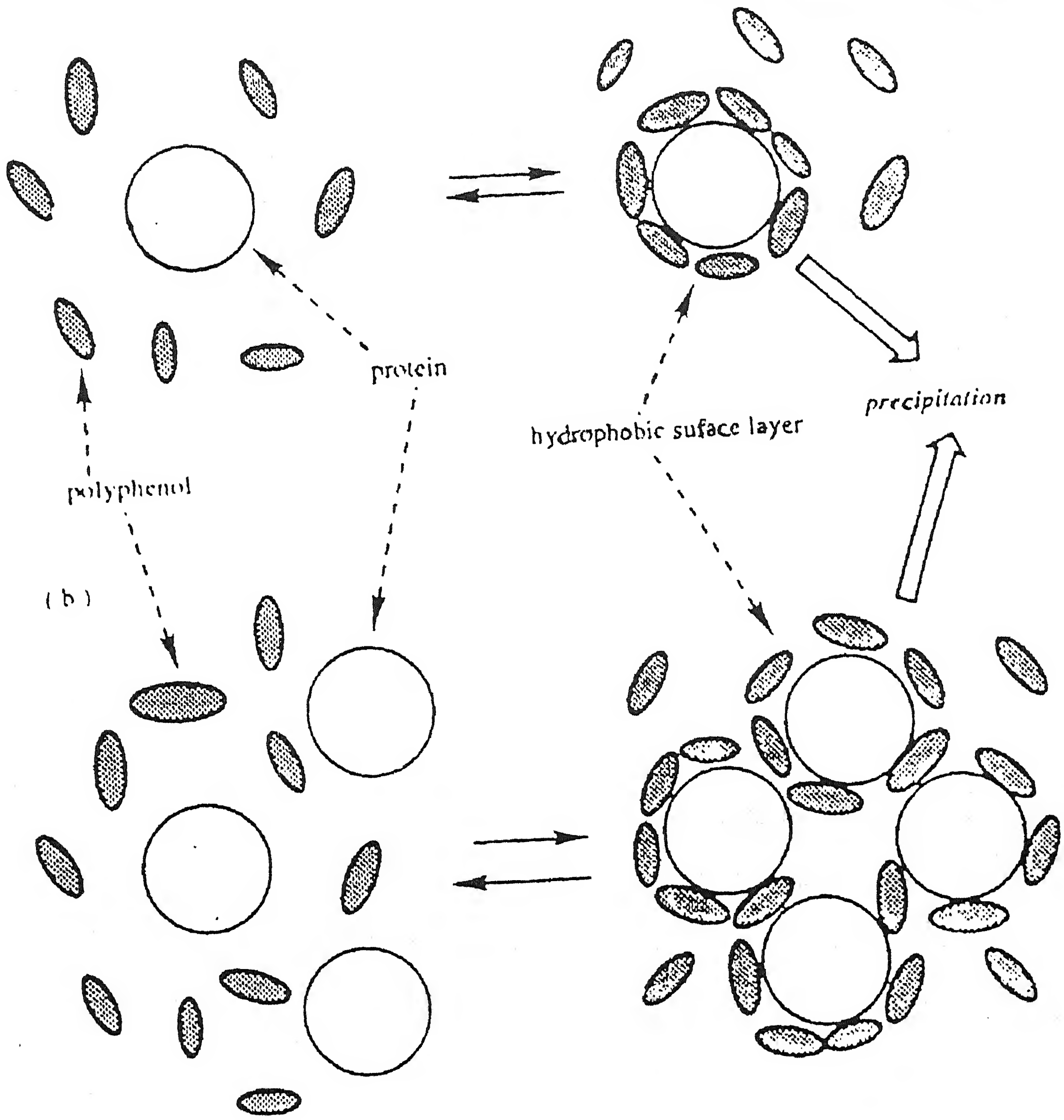

Fig 1 :- Courtsey form Haslam *et.al.*, (1999)

RESULT AND DISCUSSION

The isolated biflavonoids AP-1, AP-2, AP-3, BP-1, BP-2, and LD, from the plants under study were used for determination of protein binding capacity by using Bovine Serum Albumin Fraction(BSA V.)The results have been exhibited as μg BSA/g compound in Table-1.

All the studied compounds were polyhydroxy flavonoids in nature. Among all the biflavonoids obtained from *Albizia procera*, the highest protein binding capacity was observed in AP-3, 253.05 μg BSA/g compound followed by AP-2, 230.46 μg BSA/g compound and AP-1 198.31 μg BSA/g compound. It was noteworthy that compound AP-3 possessed molecular weight 810 with 10 OH whereas AP-2 and AP-3 were of molecular weight 808 and 766 with 8 OH and 6 OH respectively. It was well in agreement with the reason given by Beart *et.al.*, [1985] and Mangan [1988] that the protein precipitating capacity of polyphenolics depend on the number of OH groups in molecule as well as their molecular weight [Bate and Smith, 1973]. The large number of phenolic groups in the molecule provide many points of attachement with favourable steric opportunities for linkage by hydrogen bonding with the peptides of adjacent protein chains to form protein tannin complex [Gustavson, *et.al.*, 1952].

In biflavonoids BP-1 and BP-2 isolated from *Bauhinia purpurea* the protein binding capacity was 210.4 μg BSA/g compound and 199.24 μg BSA/g compound possessing molecular wt. 752 and 810 respectively. The lower protein binding capacity of BP-2 could be due to presence of sugar moieties in molecule causing conformational mobility in glucoside due to frequent intramolecular change in equitorial and axial positions [Haslam, 1977]. Beart [1988], Ojawa

et.al., [1987] and Mangan [1988] stated that the capacity to bind protein by polyphenolics depend on molecular size, conformational flexibility spatial configuration and low water solubility in the polyphenol subset.

The biflavanoid BP-1 and AP-1 had almost similar affinity with BSA protein inspite of different molecular weight and hydroxy group. Both the biflavanoid BP-1 and AP-1 had 4 OH group on the periphery which might have taken part in protein binding [Beart *et.al.*, 1985]. It was further evident from the relative higher protein binding capacity of biflavanoid molecule of LD as 201µg BSA/g with molecular weight 766 and having all 5 OH on periphery.

The discrimination were found between protein binding capacity of BP-1, 210 µg BSA/g compound possessing molecular weight 782 and 4 OH, and LD having protein binding capacity 201.81 µg BSA/g with M.W. 766 with 5 OH it might be due to hydrophobicity of BP-1, since there is no hydrogen bonding between 4 carbonyl group and 5 OH makes it less water soluble [Mori *et.al.*, 1987].

The protein biflavanoid interaction is of ecological importance because the biflavonoids diposited in plant leaves will clearly come in direct contact with the proteineous enzyme secreated by invading pathogens, and act as chemical weapon in plant defence

Table – 1

Protein binding efficiency of biflavonoids from *Albizia procera, Bauhinia purpurea Leucaena diversifolia* leaves.

S. N.	Name of Biflavonoids	Plant species	Mol. wt.	Glycoside or aglycone	Protein Binding efficacy μg BSA/g comp.	No. of substi-tution
1.	AP-1	*Albizia procera*	766	Aglycone	198.31	6 OH
2.	AP-2	*Albizia procera*	808	Aglycone	230.46	8 OH
3.	AP-3	*Albizia procera*	810	Aglycon	253.06	10 OH
4.	B.P.-1	*Bauhinia purpurea*	782	Aglycone	210.4	4 OH
5.	B.P.-2	*Bauhinia purpurea*	810	Glycoside	199.24	10 OH
6.	L.D.	*Leucaena diversifolia*	766	Aglycone	201.81	5 OH

EXPERIMENTAL

Protein binding efficiency of biflavonoids were estimated on the basis of method given by Dawra *et.al.*, [1988].

Reagents :-

1. **BSA solution** -2mg/ml in acetate buffer 0.05M. Adjust its pH 5 by adding

2 M NaOH

2. 0.2% Ponceau S in 3% trichloro acetic acid.
3. 0.2% Acetic Acid
4. 0.1N NaOH
5. **Standard Tannic acid :-**

Standard Tannic acid solution was prepared in methanol : H_2O (1:1) with equal quantity of ascorbic acid were added to minimise the oxidation during handling.

CALIBRATION CURVE WITH TANNIC ACID :-

The aliquots of tannic acid containing 50,75,100,125,150,175,200 μg/ml of tannic acid were applied on 1mm. Whatman chromatography No.1 paper at different places. The spots were allowed to dry and sprayed immediately with BSA solution until the paper was completely wet. After 30 minutes paper was washed 3 times with acetate buffer thoroughly to remove unbound tannic acid. The paper was stained with Ponceau S three times. The stained strips were washed in 0.2% acetic acid v/v until the elution of colour from the strip was ceased . The strips were air dried and stained areas were cut. Along with blank into small pieces and kept in separate test tube. The colour was eluted with 3ml of 0.1 N NaOH

followed by addition of 0.3ml of 10% acetic acid. After centrifugation the colour in the supernatant was recorded at 525nm and draw the calibration curve . (Fig-2)

DETERMINATION OF PROTEIN BINDING CAPACITY OF ISOLATED BIFLAVONOIDS :-

The known quantities of isolated biflavonoids were dissolved in MeOH. From these solutions of biflavonoid 0.1ml, 0.2ml in three replicates were applied on Whatman No.1 paper as spot corresponding 10 μg, 20 μg, quantities and followed the procedure as described above the μg BSA calculated with standard curve of tannic acid.

Standard curve with Tannic Acid

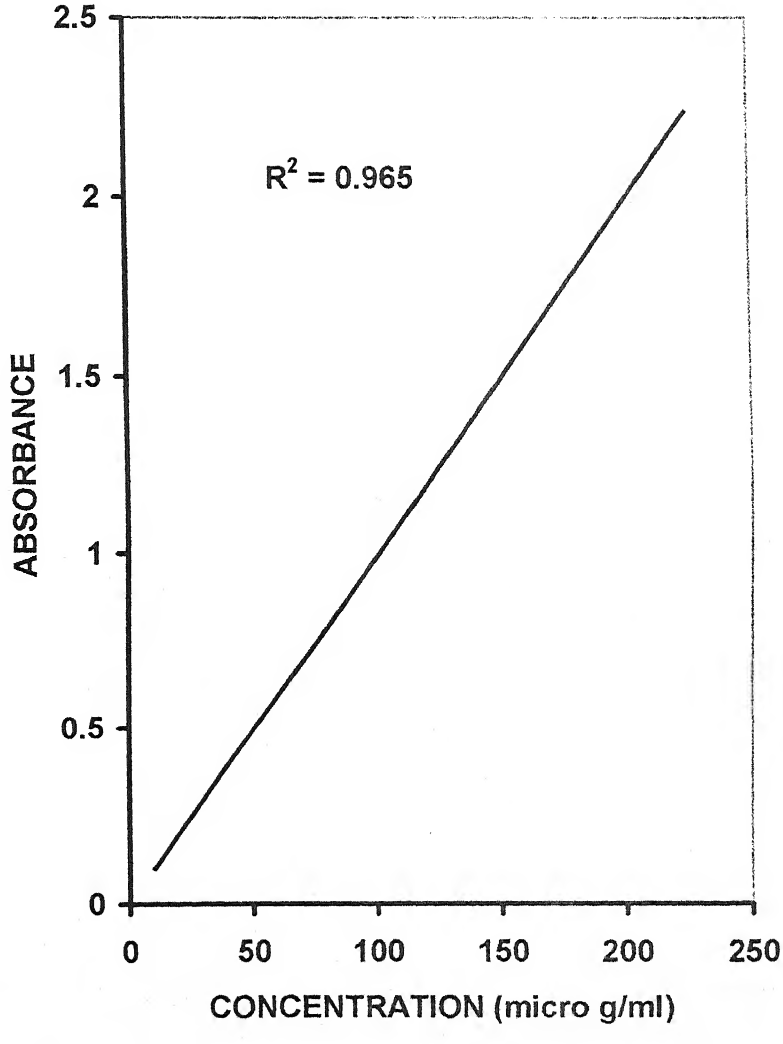

FIG - 2

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Appendices

Photocopies of Published/Accepted

and communicated paper.

Indian J. Anim. Nutr., 18(2), 113 - 118, 2001

CHEMICAL AND BIOCHEMICAL ASSESSMENT OF SOME LEGUMINOUS TREE LEAVES

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ABSTRACT

A study was conducted to assess the antinutritional factors of *Bauhinia purpurea*, *Leucaena diversifolia* and *Albizia procerra* in terms of total phenolics (TP), condensed tannin (CT)/proanthocyanidins (PA), relative degree of polymerisation (RDP), protein precipitating capacity (PPC) and protein precipitable phenolics (PPP) in relation to nutritional attributes such as crude protein, lignin, hemicellulose and IVDMD. The investigation revealed that *B.purpurea* possesses CT (195.0 mg/g) with PPC (7.438mg BSA/g) and PPP (64.94%) in comparison to that of *L.diversifolia* CT (21.07mg/g), PPC (0. 129mg BSA/g), PPP(51.73%) and *Albizia procerra* CT (16.48mg/g), PPC (0.6945mg BSA g), PPP (41.26%). **These fodder leaves could be ranked for their utilization *L.diversifolia* > *A.procerra* > *B.purpurea* for suitable dilution in other feed.**

Key words : Proanthocyanidines, Condensed tannins, Protein precipitating capacity, Flavan-3 ol, Tree leaves

The leaves of tree and shrubs are a major feed resource for domestic ruminants in arid, semi arid and temperate regions. The top feeds are used as either freshly lopped or in dried conserved state. Despite their high protein content and energy they often contain anti-nutritional factors like tannins, saponins, alkaloids etc. which might restrict the efficient utilization of their nutrients. Phenolics are the most widely occuring constituent in angiosperms (Harborne 1992). There are a number of indigenous leguminous tree in native floara which may be useful to bridge the gap of demand and supply for the domestic ruminants. Therefore, prior to their inclusion in rations it is essential to examine thoroughly the nature and status of phenolics or other toxicants. With this view we report chemical and biochemical nature of three leguminous fodder tree leaves viz. *Bauhinia purpurea, Leucaena diversifolia* and *Albizia procerra.*

MATERIALS AND METHODS

Leaves of *A. procerra, B. purpurea* and *L. diversifolia,* collected from the CR farm of the Indian Grassland and Fodder Research Institute, were freeze dried and ground to pass through 1 mm. s[illegible] analysis.

* *Chemistry D[illegible]rtment, Bipin [illegible], Jhansi (U.P.)*

Chemical assessment of phenolics

Total phenolics (TP) were analysed as per Prussian blue method (Price and Butler 1977) using 300 mg of representative samples of leaves for extraction with 20 ml methanol for 24 hrs. The extract volume was made up to 50 ml with methanol and an aliquot (0.1 ml) was taken for colour development with 3.0 ml 0.05M $FeCl_3$ (in 0.01 N HCl) and 3 ml 0.08 M K_3 [Fe $(CN)_6$] in 60 ml distilled water. Absorbance at 725 nm was recorded after 25 min. The results were calculated as catechin equivalent and tannic acid equivalent from the standard curve for catechin (Sigma) and tannic acid (E.Merck), respectively.

The vanillin/HCl assay (Broadhurst and Jones, 1978) was used for determination of condensed tannins (CT). CT were estimated by extracting known quantities of leaves in 30 ml of 70% acetone and extracts were washed with diethyl ether and made up to a fixed volume. One ml of aliquot was treated with 5ml vanillin/ HCl reagent and absorbance was recorded at 510 nm. The results were expressed as catechin equivalent with standard curve prepared from catechin.

70% Acetone extract of leaf samples (0.5 ml) were taken in test tube with 3 ml of n-Butanol : HCl (95:5 v/v) and 0.1 ml Ferric reagent (2% Ferric Ammonium Sulphate in 2 N HCl). The reaction mixture were kept at 100°C in a water bath for 60 min. and cooled to note the absorbance at 550 nm as described by Porter *et al.* (1986)

Biochemical assessment of phenolics

The biochemical value of phenolics were assessed by preparing tannin protein complex (TPC) with Bovine Serum Albumin (Fraction V). For this purpose the representative samples were extracted with 50% methanol and prepared as given by Martin and Martin (1982) and proceeded as per Makkar *et al.* (1987)

Tannin assay in tannin protein complex (TPC)

One ml. of aliquot prepared by dissolving TPC complex in 1% SDS solution was mixed with 3 ml SDS - Triethanolamine (1% SDS w/v and 7% TEA) and treated with 1ml $FeCl_3$ (0.01 m $FeCl_3$ in 0.1 N HCl) and thoroughly mixed, to record absorbance at 510 nm after 20 min as described by Hagermann and Butler (1978). The tannin was calculated as tannic acid equivalent from standard curve of tannic acid.

Protein in Tannin Protein Complex

0.1 ml of aliquot (TPC dissolved in SDS) was dried at 80±10°C, followed by alkaline hydrolysis (13.5 N NaOH) colour was developed with ninhydrin (Makkar *et al.* 1987).

Determination of total phenolics in 50% MeOH extract

Total phenolics in 50% methanolic extract of the plants were estimated to determine the protein precipitable phenolics as per the protocol given by Makkar (1994).

The relative degree of polymerisation was estimated as described by Butler (1982). Specific activity of tannins was calculated by determining the ratio of protein precipitating capacity to protein percipitable phenolics (Hagerman and Butler, 1989).

Determination of mimosine

Fresh leaves of *L.diversifolia* (1 g) were processed as per the method of Brewbacker and Kays (1981) and an aliquot (2 ml) was treated with 1 ml of $FeCl_3$ (0.8% in 0.1 N HCl). Absorbance was recorded at 535 nm and compared with standard curve with pure mimosine (Sigma).

Nutritional Assessment

The crude protein content in leaves was determined by Kjeldahl technique. The cell wall fractions viz. ADF, NDF and lignin were assayed as per Goering and Van Soest (1970). The *in vitro* dry matter disappearance of the feed was determined (Tilley and Terry 1963) using inoculum from crosbreed steer fed on wheat straw and concentrate as per recommendation. The MS Excel was used for statistical analysis for determination of corelationships.

Table 1 Content of condensed tannin, proanthocyanidins, total phenolics, protein precipitation capacity, specific activity and degree of polymerisation in three leguminous tree leaves

Phenolic attributes	*L. diversifolia*	*B.purpurea*	*A. procerra*
CT/PA mg/g (Vanillin/HCl)	21.07±0.37	195.0±2.24	16.48±0.002
CT/PA % (Butanol/HCL)	0.623±0.001	6.083±0.25	0.253±0.001
Total phenolics mg/g (Catechin equivalent)	42.53±0.23	154.73±1.28	25.053±0.001
Total phenolics mg/g (Tanninc acid equivalent)	23.36±1.8	102.10±1.28	40.00±0.72
Total phenolics by SDS method (Y) %	5.54±0.12	10.28±0.044	0.511±0.005
Phenol in tannin protein complex (X) (mg/g)	3.01±0.90	6.63±1.21	2.10±0.06
Protein in tannin protein complex (Z) mg/g	0.129±0.009	7.43±0.14	0.694±0.005
Specific Activity (Z/X)	0.052±0.003	1.12±0.14	0.33±0.025
Protein pricipitable phenols. (X/Y)* 100(%)	51.73±2.9	64.94±2.8	41.26±1.57
Relative degree of polymerisation	[illegible]	1.366±0.02	0.785±0.006

RESULTS AND DISCUSSION

The chemical and biochemical nature for the phenolics are exhibited in table 1 and nutritional attributes of these three leaves are summarized in table 2. The maximum concentration of CT (195 mg/g as catechin equivalent) and TP 154.73 mg/g as tannic acid equivalent, 102 mg/g as tannic acid equivalent were noted in *B.purpurea,* whereas in *L. diversifolia* the CT (21.07 mg/g) was higher than that of *A. procerra* (16.89 mg/g). The TP estimated as catechin equivalent was higher in *L.diversifolia* (42.53mg/g catechin equivalent) than that of *A.procerra* (25.05mg/g) while the total phenol as tannic acid equivalent were in reverse order in these tree leaves. Since tanic acid forms more stable standard and more ability to reduce Fe^{+3} than that of catechin (Deshpandey and Cheryan, 1987), therefore, the values obtained as tannic acid equivalent were considered nearer to precison than that of catechin equivalent. The flavan-3-ol determined as proanthocyanidin (Porter 1994) (PA), were found highest in *B.purpurea* (6.0 unit) followed by *L.diversifolia* (0.623) & (0.252) in *A.procerra.*

The biochemical assessment of the phenolics in these tree leaves revealed that the phenolics present in *B.purpurea* possesed highest protein precipitating capacity (PPC) (7.43mg BSA/g) and relative degree of polymerisation (RDP) (1.3). The PPC (0.694) and relative degree of polymerisation (0.785) of *A.procerra* was higher than that of *L.diversifolia* (PPC 0. 129, RDP 0.325). The protein precipitable phenolics in *L.diversifolia, B.purpurea* and *A.procerra* were 51.73, 64.94 and 41.26%, respectively.

Table 2 Nutritional attributes of three fodder tree leaves (% DM basis)

Attributes	*L.diversifolia*	*B.purpurea*	*A.procerra*
Crude protein	24.86±0.37	28.23±0.32	22.08±0.74
NDF	35.09±0.21	43.17±0.42	43.87±0.229
ADF	26.81±0.23	37.70±0.28	39.02±0.232
Lignin	11.11±0.26	14.74±0.28	9.05±0.188
Hemicellulose	8.17	5.46	4.85
IVDMD	50.67±0.30	38.17±0.21	51.59±0.49

Specific activity of tannins, an index of protein bound per unit of phenolic molecule was highest in *B.puruputea* (1.12) and followed by *A.procerra* (0.33) and *L.diversifolia* (0.052) suggesting *B.purpurea,* has stronger affinity for protein molecules than *A.procerra* and *L.diversifolia.* Therefore, the PPC and RDP of tannin molecule in any material is not only indicative of protein phenolic interaction but the SA is also taken in account to assess the phenolics. A highly positive significant correlation coefficient was shown between PPC and PPP in all these three fodder trees *L.diversifolia* $r = 0.98$ ($P<0.05$, $n = 5$) *B.purpurea* $r = 0.93$ ($P<0.05$, $n = 5$) *A.procerra* $r = 0.88$ ($P<0.05$, $n = 5$). The inverse relationship

between PPC and RDP in all the three species were well in agreement with earlier workers (Goldstein and Swain 1963; Jones *et al.*, 1976; Butler, 1982; Kumar, 1983) that the capacity of tannin to bind protein was related to the molecular size of the tannin as the surface area of the molecule was decreased with polymerisation (Makkar *et al.*,1987).

Although from the nutritional point of view all the three tree leaves under investigation were of good protein value containing crude protein ranging between 22-28% yet *B.purpurea* had lowest IVDMD (38.17%) whereas *L.diversifolia* and *A.procerra* were found to possess 50.67 and 51.50% IVDMD respectively. Based on the data for *B.purpurea, L.diversifolia* and *A.procerra* the correlation between IVDMD and proanthocyanidin level (BuOH-HCl), mainly representing flavan 3-4 diol, were -0.65, -0.696 and -0.386 ($P>0.05$, $n = 5$) respectively. There was a negative correlation between IVDMD and total phenolics as -0.847 ($P<0.05$, $n = 5$), - 0.241 ($P>0.05$, $n = 5$), -0.348 ($P>0.05$, $n = 5$) for *B.purpurea, L.diversifolia* and *A.procerra,* respectively. Moreover lignin content was also found to negatively correlated with IVDMD as usual. Beside these, *L.diversifolia* was found to contain mimosine, an another antinutrient phonolic, non-protein amino acid (1.711% DM basis). This clearly indicated the role of phenolics in lowering the digestibility of woody perennials.

Therefore, it is much warranted that the tree leaves should be utilized after overcoming the effects of phenolics by judicious computations with feed. The nature of tannins should also be defined precisely to establish the structure activity relationship which would help in developing suitable technique to mitigate the problems in future.

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